NASA SP-165

BIOREGENERATIVE SYSTEMS

A CONFERENCE HELD IN

Washington, D.C.

November 15-16, 1966

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

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Foreword

Conferences such as this one bring together scientists dealing with a fundamental problem for uninhibited and constructive discussion of each other's contributions to a major scientific endeavor. Often groups of investigators work independently on specific aspects of a broad program encompassing a single objective. An example is the complex, multidisciplinary research program dealing with the organism *Hydrogenomonas* and its practical utility in the chemosynthetic approach to a closed ecologic bioregenerative life-support system. Through these conferences, the solution of problems can be more closely interwoven with more integration and dissemination among investigators of the problems and trends in this type of vast research. This conference was the second in this series. Unfortunately, we were unable to publish the proceedings of the first meeting in Columbus, Ohio, 1965.

Looking back to our first get-together, we found ourselves acting as a self-contained appraisal and advisory panel. In the course of our discussion, we interposed reservations about specific direction of the program, brought to light major biologic parameters that temporarily occluded our mission, and identified the pathways by which we could proceed to the current state of progress we shared during our second conference.

The second, and equally important, purpose of these conferences is coordination, through candid and leisurely exchange of information; it is virtually impossible through written communication. Our sincere appreciation is extended to the participants. The excellence of these conferences is attributed to their diligence and dedication to the solution of a complex biologic problem.

JOSEPH F. SAUNDERS
Chief, Environmental Biology Bioscience Programs
Office of Space Science and Applications
National Aeronautics and Space Administration

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Introduction

JOSEPH F. SAUNDERS
Office of Space Science and Applications, NASA
Washington, D.C.

It is necessary to define the objective and the goal of this conference. A distinction exists, if they are defined as according to Dr. Robert Seamans, NASA Deputy Administrator: "A goal—and usually in NASA we speak of such a goal as a 'mission objective'—may be described as something to be done in a given way, at a given time, for a given cost. A broad objective, on the other hand, is what we hope to accomplish as a result of reaching a series of specific goals." As indicated in the agenda, our broad objective for the future is a bioregenerative life-support system. The series of specific goals is represented in the various research tasks which you, as grantees and contractors, are pursuing in what we like to refer to as a systematic and concerted multidisciplinary effort.

When we asked for your participation, we indicated that we wished to confer on the progress that has been achieved in the *Hydrogenomonas*-chemosynthesis and photosynthesis approaches to a bioregenerative closed ecologic system. We were hoping to encourage an exchange between those scientists working with that system and those pursuing the photosynthetic approach. Our final goal evolved about a discussion of the problems involved in these systems, avenues of approach to solutions, and the potential of either a single or mixed system for fulfilling a mission objective—long-term life support.

Again, referring to the excellent list of topics to be presented and discussed, we should be able simply to put the pieces together and solve a perplexing puzzle. Unfortunately, this is not the case. During my 12 years with the Office of Naval Research, I was constantly plagued and harrassed by the expression "man is the weakest link in the weapons system." I hope this will not be true for the biologic organism in the bioregenerative system. I hope, also, that we can elaborate upon certain concepts and research results to answer some of the questions we must ask. These may possibly include:

1. In the *Hydrogenomonas*-chemosynthesis system we wish to know (a) is there an inherent danger of damage to the enzyme system, hydrogenase, by virtue of the molecular oxygen in the medium; (b) regarding *Hydrogenomonas*-bacteriophage relationships, are there differences between heterotrophs and autotrophs, what is the extent of lysogeny, and what are the potential dangers in a continuous culture system; (c) what are the factors required for protein synthesis; (d) are neutral fats and polysaccharides formed and what are their characteristics so that some factor can be ascribed from the nutritional point of view; (e) are we myopic in concentrating on aerobes instead of exploiting anaerobes such as *Clostridium aceticum* singly or in combination with aerobes such as *Hydrogenomonas eutropha;* and (f) what is the genetic stability of such cultures?

2. Looking at both processes, (a) what are the advantages and disadvantages of each system; (b) is there any mutuality or commonality (for example) at the molecular level for energy collection and utilization, electron transport systems, etc.; are the energy collection and conversion sites similar, molecularly; (c) what are the kinetics of the various chemical reactions taking place in the closed ecologic system; (d) what are the trace element requirements for coenzyme function; (e) are there metabolic endotoxins or toxins in human waste materials that could wreak havoc in the system; (f) is membrane function and resistance to exogenous poisons a problem area; (g) what must we know

about excited states and intermediates of assimilates such as urea; and (h) can we think of a combination of chemosynthesis-photosynthesis?

3. Are we technologically on the verge of an engineering design to test the feasibility of a bioregenerative life-support system; can we decree with a high degree of certainty and confidence that these systems could withstand the rigors of space flight such as acceleration and vibration; is there any danger to them from such space environment factors as radiation and null magnetism?

I added the latter merely to provoke such comments as are apropos. However, your deliberations may be pertinent to what the next speaker has to say. I do hope, however, that some of the foregoing problems will be given attention in the presentations and considerable thought during the summary session.

It is often said that when one inherits a scientific program, one not only takes on the good but must live with the bad. My comments concerning this are that we would not be assembled here today to confer on one of the dynamic bioscience task areas of NASA, had it not been for the foresight and direction of Dr. Dale W. Jenkins. I do not wish to overdo the accolades, but I must say that this NASA program will be synonymous with the name of Dr. Jenkins. With his continued support, I hope to carry it to fruition, perhaps as a future Biosatellite flight experiment for a pilot study. It is now my privilege to present the Assistant Director for Science of our Bioscience Programs Office, Dr. Dale Jenkins.

Bioregenerative Life-Support Systems

DALE W. JENKINS
Office of Space Science and Applications, NASA
Washington, D.C.

Closed ecological life-support systems are one of the most difficult scientific and engineering tasks in the space program. Manned space flight of long duration requires a complete life-support system able to supply all the oxygen, food, and water; to remove all excess carbon dioxide, water vapor, and human body wastes (table I); to maintain the oxygen, carbon dioxide, barometric pressure, and temperature at a suitable level; and to remove any accumulated toxic products and noxious odors. In the spacecraft, a human being is confined in a restricted environment where it is necessary to miniaturize a completely balanced microcosm or closed ecological system. This is an enormous biological and bioengineering problem. Weight, size, simplicity, and efficiency of operation, and, in particular, reliability are important factors. The disciplines involved in such systems include biology and microbiology, cryogenic fluid handling in low gravity, heat transfer, and thermal integration with other systems, such as power. The physiological, psychological, and sociological problems of man must also be considered.

TABLE I.—*Average Daily Metabolic Data for a 70-kg, 25-Year-Old Astronaut With Normal Spacecrew Activity* [a]

Item	Data
O_2 uptake	0.862 kg.
CO_2 output	1.056 kg.
Drinking H_2O	2.5 liters.
Food-rehydrating H_2O	1.0 liter.
Food	3000 kcal.
Water output:	
Urine	1.6 liters.
Respiration and perspiration	2.13 liters.
Feces	0.09 kg.
Total heat output	11 100 Btu.

[a] After Talbot (ref. 1)

There have been about 15 years of fairly intensive research on algae, with occasional research studies on higher plants, and about 4 years of research on *Hydrogenomonas* bacteria and electrolysis. During this time, no efficient and reliable system capable of continued operation has been developed.

Review of the research and development contracts of NASA, the Air Force, and other government agencies indicates that about $30 million have been spent on research in photosynthetic bioregenerative systems during a period of 12 to 15 years. NASA has supported limited research ($1 million) on algae and higher plants to determine their potential for bioregenerative systems. Research programs on continuous culture of algae and photosynthesis have been supported for about 6 years at the University of Maryland, University of Minnesota, and the Research Institute for Advanced Study (RIAS). The Air Force has been quite heavily involved in research on photosynthetic systems. It was informally agreed that the Air Force would mainly support the photosynthetic systems and NASA the bacterial systems, to prevent duplication of effort. In the *Hydrogenomonas* research and development program, less than $1 million has been spent by NASA. The present program support is about $0.3 million per year. Other bacterial systems of interest and with a promising potential include *Methanobacter* in combination with *Pseudomonas* bacteria, suggested by Dr. R. Humphreys, University of Pennsylvania.

The major questions, often asked, are: will bioregenerative systems be used, when, and to what extent? Several answers are given. Some people have talked about farming on Mars or the Moon. Others about bioregenerative systems for two or three men for 2- or 3-month missions. In many publications, tradeoffs of stored

versus regenerative systems are considered for a variety of missions, ranging from a few men for a few months to a number of men for several years. Present plans in NASA for manned missions indicate a possible first requirement for bioregenerative systems around 1985. A manned space station holding possibly nine men for several years is being considered for about 1973. A manned lunar landing is planned by about 1970, followed perhaps by lunar exploration programs. (A lunar base has been proposed, but it is not a firm program.) There seems to be no need for a bioregenerative system in currently discussed lunar bases. As yet, no serious plans are being made for a probably large number of people for a long stay on the Moon.

A manned fly-by to Mars with a surface sample return has been discussed for 1975; this would require about 700 days with four or more men flying by Mars in an ellipse into the asteroid belt and back to Earth. It is doubtful that the required life-support system for such a mission would be ready by 1975. A manned Mars lander is being considered for the 1980's. There are three possible types of flight. The first is at opposition, in which the two planets are fairly close together in their orbits around the Sun. This includes a direct transfer mission from the Earth to Mars, a stay time of about 50 days, and then return. The total time would vary from about 400 to 600 days, depending on the year. In the conjunction-type mission there is a transfer from Earth to Mars, taking about the same period of time, but involving a stay time of about 400 to 500 days, and then a return when Mars comes around in opposition again. This conjunction-type mission would take from 900 to 1000 days. The other type of Mars manned mission is the Venus swing-by, flying directly from Earth to Mars with a stay time of about 10 to 50 days, depending on the time period, and then swinging in toward Venus and using its gravitational pull to swing around and come back to Earth. This mission requires about 400 to 600 days, depending on the date.

These missions last from 400 to 1000 days. Most studies have shown that men confined for long periods should number not less than six, preferably eight—for psychological and sociological reasons.

A company of eight to 10 men shut up for 400 to 1000 days would require regenerative life-support systems. Bioregenerative systems would have to be weighed in comparison with the chemoregenerative systems that are being developed by the NASA Office of Advanced Research and Technology.

The first potential mission requiring a bioregenerative system would be a manned Mars landing in the 1980's. The 1984 opposition is one of the best from the standpoint of shortest distance in time and probable availability of propulsion systems and technology. With a 1984 goal, flight-qualified life-support equipment would have to be ready to install in a flight system in 1979. By about 1974, completely designed life-support hardware should be ready for quality and reliability testing. This equipment would have to be tried out in a space station under weightlessness or low gravity. (No decision has yet been made whether partial gravity is required in a space station.)

Two types of biological regenerative systems have been proposed. The photosynthetic system involves the use of algae or higher plants and requires the introduction of light energy with carbon dioxide and water to produce oxygen, cells, and water. Another system, proposed in 1961, involves the electrolysis of water and the concurrent use of *Hydrogenomonas* bacteria that take up hydrogen, carbon dioxide, and urine and provide water and bacterial cells. The difference between the two biosynthetic processes—photosynthesis and chemosynthesis ("dark" synthesis)—is not in the synthesis itself, but in how the hydrogen is made available from water to reduce the hydrogen carrier. For photosynthesis, light energy is used to split the water (photolysis). If only artificial light is available, a heavy loss (about 80 percent) must be taken into account in converting electricity into visible light.

The photosynthetic bioregenerative system has been studied extensively, with major emphasis on aquatic algae—which are the most efficient plants for closed ecological systems. The use of

algae has been critically reviewed and the results of all investigators compared (ref. 2). Using fluorescent light, the most efficient system for the support of one man requires 100 liters of algae with 10.4 kw continuous and 12 square meters of illumination surface (ref. 3). Using a GE high-light intensity Quartzline lamp, the most efficient system requires 43 liters, 48.0 kw continuous, and 1.7 square meters of illumination surface (ref. 4).

It has been amply demonstrated that *Chlorella* can be used in a closed ecological system to maintain animals such as mice or a monkey. The algal gas exchanger has the capability of: (a) efficiently supplying all required oxygen; (b) rapidly and effectively removing all carbon dioxide; (c) removing excess water vapor from the air; (d) removing noxious and toxic odors from the air; (e) utilizing waste water from washing; (f) utilizing urine; (g) utilizing feces and other organic and nitrogenous wastes; (h) recycling water to provide clean water for drinking and washing; (i) supplying food in the form of algae or algal products; (j) supplying food to animals to produce animal fat and protein. The use of algae for supplying oxygen, food, and water, and removing carbon dioxide, water vapor, and odors has been considered by many authors for use in spacecraft and space stations, and for establishing bases on the Moon or Mars.

NASA has supported the development of the *Hydrogenomonas*-electrolysis bioregenerative life-support system. This system combines the electrolysis of water with the growth of *Hydrogenomonas eutropha* bacteria. Water is split into oxygen (supplied to the astronaut and bacteria) and hydrogen (supplied to the *Hydrogenomonas* bacteria together with carbon dioxide and urine from the astronaut). Water of respiration from the astronaut and water produced by the bacteria are used in electrolysis. The bacteria can be a potential partial food source. The electrolysis system has been developed to operate under conditions of weightlessness with about 80 percent efficiency. This electrolysis-bacterial system requires no light energy and is potentially more efficient than the best algal or higher plant bioregenerative systems on the basis of electrical power requirements. At present, comparison by weight and volume indicates the electrolysis-bacterial system as the more efficient.

The chemosynthetic conversion is carried out by the hydrogen bacteria. In the enzymatic cleavage of molecular hydrogen, supplied from the electrolysis of water, energy is made available for biosynthesis. The generation of this "biological energy" is mediated by a stable enzyme, hydrogenase, which is present in most hydrogen bacteria. On the average, a cleavage of 4 moles of H_2 is required for the conversion of 1 mole of CO_2 (the hourly production of a man). The removal of this amount of CO_2 would thus require the cleavage of 4 moles of water. In addition, to supply oxygen for human respiration (at a rate of 1 mole of O_2 per hour), requires the cleavage of 2 additional moles of water. Therefore, the chemosynthetic regeneration and human respiration together would require, on the average, the splitting of 6 moles of water per hour.

Figure 1 illustrates the material balances between the electrolysis, bacteria, and astronaut. Comparison with the average metabolic data of an astronaut as recorded in table I shows that the CO_2 of the astronaut and the bacteria are balanced at 1.056 kg per day. The water relations are not completely balanced, but are fairly close. In recent NASA-supported research, the amount of culture medium has been estimated by improved cultivation methods under the right conditions.

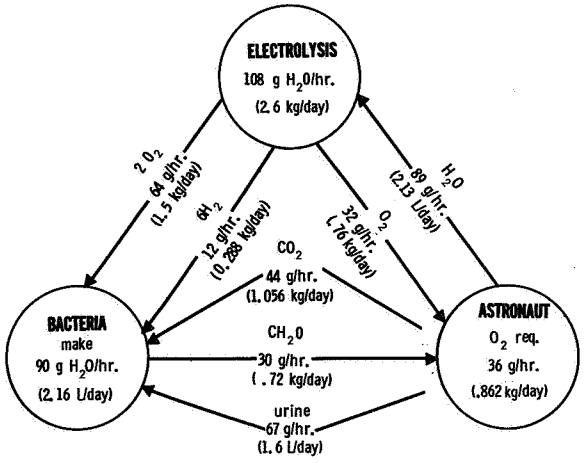

FIGURE 1.—Diagram of material balances in the electrolysis Hydrogenomonas life-support system.

For batch culture, the data show that from 10 to 66 liters would be required per man with the best practical estimate of 20 liters with 9- to 10-g dry weight of bacteria per liter (ref. 5). For continuous culture, the present data, using the turbidostat, indicate a demand of some 30 liters of suspension, and a volume of 20 liters (approximately 10-g dry weight of bacteria per liter) as a realistic goal. At present, a density of 7-g dry weight per liter has been obtained (ref. 6).

Growth of hydrogen bacteria as a batch culture, after an initial period of adjustment, becomes steady and rapid during the exponential growth phase. This steady state of growth is temporary and ceases when nutrient substrate or gas concentrations drop to limiting values. For long periods, a continual supply of nutrients must be provided. Growth then occurs under steady-state conditions for prolonged periods; and such variable factors as nutrient concentration, oxygen, pH, and metabolic products (which eventually change during the growth cycle in batch culture) are all maintained constant in continuous culture.

Use of an electrolysis-hydrogen bacterial life-support system requires continuous bacterial culture and integration with the electrolysis. The electrolysis system formed a separate unit; the hydrogen and oxygen gases were fed into the medium alternately with carbon dioxide.

Two methods can be used for control of continuous cultures. The turbidostat is regulated by medium input, and cell concentration is controlled by optically sensing the turbidity of the culture. Organisms grow at the maximum rate characteristic of the organism and the environmental conditions applied. A disadvantage of the turbidostat is that all nutrient concentrations in the culture chamber are necessarily higher than the minimum, causing inefficient nutrient consumption. The turbidostat system for continuous culture of *Hydrogenomonas* bacteria, developed by Battelle Memorial Institute, includes electrolysis of water in a separate unit. Hydrogen and oxygen are fed separately up to the point of injection into the culture vessel, and the mixed volume is kept very small to minimize any possibility of explosion.

In the chemostat, growth of the organisms is limited by maintaining an essential nutrient concentration below optimum. A constant feed of medium (with one nutrient in limiting concentration) with constant removal of culture at the same rate is used to achieve the steady state. The dilution rate is set at a predetermined value and the microbe population is allowed to find its own level. This constitutes a self-regulating system and allows selection of a desired growth rate and population age.

Both continuous culture approaches are being studied with NASA support. The turbidostat offers the greatest potential for greatest efficiency in weight and volume, but is less efficient in the use of nutrient materials and has greater engineering complexity. The chemostat is less efficient in weight and volume, but is simpler and more reliable. *Hydrogenomonas eutropha* has been grown in 15-liter batch cultures and in 2.1-liter continuous cultures. A 20-liter continuous culture to balance the requirements of a man is under development.

Potential problems in large-scale continuous bacteria production include assuring genetic stability, the prevention or control of virus phage and bacterial contamination, and the prevention of heterotrophic growth. Research is progressing on these possible problems. Genetics of hydrogen bacteria and phage infection have been studied by DeCicco (ref. 7). When the bacteria are exposed to organic material (in the urine), they change from autotrophic to heterotrophic growth and do not utilize hydrogen. The intermediary metabolism of the bacteria is being studied, with radioisotope labeling. These problems cause significant effects and must be eliminated or controlled. *Hydrogenomonas* bacteria may be used for food in at least part of the astronaut's diet. The washed bacteria have a mild taste and are being studied for their total energy content, protein and lipid digestibility, and vitamin content. Carbon and nitrogen balances and respiratory quotient are being measured in animals fed on the bacteria and will be reported by Dr. Calloway.

Table II gives the power and weight requirements for both chemical and biological regenerative life-support systems. These should be considered tentative best estimates based on present

TABLE II.—*Requirements for Regenerative Life-Support Systems*

System	Requirements/man [a]		3-man [b] (270 man-day mission)	
	Weight (kg)	Power (kw)	Weight (kg)	Power (kw)
Partial chemoregenerative	------	-------	[d]332	1. 75
LiOH	125	1. 40	------	------
NaOH	155	7. 68	------	------
CO_2-H_2	34	0. 36	------	------
Full bioregenerative				
Algae—artificial illumination	116	[c]10. 40	591	25. 00
Algae—solar illumination	103	1. 70	356	0. 60
Electrolysis *Hydrogenomonas*	55	[b]0. 75	129	2. 60

[a] After Del Duca et al. (ref. 9).
[b] After Bongers and Kok (ref. 8).
[c] After Ward et al. (ref. 3).
[d] Includes instrumentation and food storage.

data. The application of recent data on bioregenerative systems to spacecraft systems has been studied by Bongers and Kok (ref. 8) who put this favorable-looking electrolysis–*Hydrogenomonas* system in proper perspective:

The bioregenerative systems are more or less in a transitory phase between research and development. The power data can be considered fairly accurate, at least within ±20%. The postulated weight data, however, represent approximations, particularly with respect to auxiliary equipment and construction materials. Also omitted are the weight penalties most probably involved in the processing of the solid output of the exchangers, elegantly defined as potential food. Further research is required in this area to evaluate the regenerative systems, especially the bacteria, with respect to this potential. Furthermore, as yet there is no experimental proof that the growth rates of the heavy bacterial suspensions can be realized in a large design, determined on a relatively small scale with fairly precise control of physiological conditions and gas exchange. This aspect may affect considerably the weight involved in a chemosynthetic balanced system. Nevertheless, at present, this approach still seems most promising.

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COMMENTS

Dr. TSUCHIYA. How is the power requirement for electrolysis calculated? From distilled water or urine?

Dr. JENKINS. The calculations are based on water, not urine.

Dr. TSUCHIYA. If there is organic matter in the system, the efficiency of meeting the power requirements drops off.

Dr. JENKINS. In the case of *Hydrogenomonas*, the urine would go to the culture medium, which is separate from the electrolysis unit, and only the water of respiration and water from fuel cells would be used for electrolysis.

Dr. TSUCHIYA. Is the fuel cell water pure enough for electrolysis?

Dr. SAUNDERS. Yes, by virtue of a filtration system in the fuel cell—resin columns and millipore filters.

Dr. JENKINS. You can drink fuel cell water. We will use it in the 30-day Biosatellite for the primate.

Dr. TSUCHIYA. Using the old-time technology, at least, they used to put in a fair amount of caustic. Thus, you will have to take care of that, too, will you not?

Dr. JENKINS. That makes the electrolysis system most efficient, according to the papers I have seen. But only pure oxygen and pure hydrogen are evolved during electrolysis, and the caustic is left in the water and does not go in with the cells. In the system that we foresee, the growth of the organisms is completely separate from the electrolysis unit.

I would like to finish by pointing out that there have been a number of conferences held—I think most of you know of these. The Air Force published one of these dealing with biologistics for space systems. This was followed 2 years later by a conference held by NASA on nutrition and related waste problems in space, and there were some papers presented on bioregenerative systems. Perhaps the most critical report that has come out recently is by Miller and Ward on the Algal Bioregenerative Systems. I think all of you have seen this. It was published in *Industrial Microbiology*, and summarizes all of the algal work all over the world. It is really an excellent treatise compiling all of the data in a system of figures so that you can actually compare different algal systems. The Air Force has also published a bibliography on bioregenerative systems for extraterrestrial habitation. In the NASA-published document, "Significant Achievements in Space Bioscience," there is a summary on bioregenerative systems. It has also been extremely interesting to see what the Russians are doing. I would like to finish with a few comments about their program.

I have talked with a number of them. One of the outstanding scientists in the bioregenerative area is Dr. Andre Rubin, whose specialty is photosynthesis. A report has been published by the Aerospace Technology Division, Library of Congress, which summarizes the work in the Soviet Union, particularly with *Chlorella* and higher plants. A Life-Support System photosynthesis report of December 1965 gives a thorough summary of the work going on in Russia on photosynthesis.

I summarized the *Hydrogenomonas* work of NASA in Athens in 1966. [Jenkins, D. W.: Electrolysis-Hydrogenomonas Bacterial Bioregenerative Life Support Sytsem. Proc. XVI Intern. Astronautical Congress, Athens 1966, pp. 229–246.] Dr. Gazenko stated that Russia had not done any work on *Hydrogenomonas*, and was interested in obtaining samples of *Hydrogenomonas* for them to look into the system. But they have done a great deal of work on *Chlorella* and quite a bit on higher plants.

Dr. SAUNDERS. Our program has been divided into three main technical sessions and a summary session. The first session is on the biochemistry and physiology of hydrogen bacteria. Dr. Bongers will begin that session with his paper on Chemoautotrophic Metabolism.

CHEMOSYNTHESIS—*HYDROGENOMONAS* SYSTEM

JOSEPH F. SAUNDERS, *Chairman*

Chemosynthetic Metabolism of *Hydrogenomonads*

LEONARD BONGERS AND JOHN C. MEDICI
Research Institute for Advanced Studies
Baltimore, Maryland

Bacteria that can synthesize all their cell constitutents, with carbon dioxide as the sole carbon source, are considered autotrophs. These organisms can be subdivided into two groups, depending on the nature of the primary source of energy used for the conversion processes. The organisms that depend on the oxidation of inorganic compounds as primary energy source are the chemoautotrophic, or the chemolithotrophic bacteria. The organisms that utilize light energy for the reduction processes are the photosynthetic or photolithotrophic bacteria. Representatives of both groups, including algae, are listed in table I, together with the energy made available in the combustion or energy-yielding reaction. The autotrophic metabolism of these miscroorganisms could conceivably be applied to balance the heterotrophic metabolism of man in a closed environment; the photosynthetic algae and the hydrogen bacteria are considered promising candidates.

Criteria for the selection of a biological subsystem for food and oxygen regeneration involve parameters such as reliability, overall size, weight and energy requirement. Hydrogen bacteria (e.g. *H. eutropha*, *H. facilis*, *H. ruhlandii*, *H. 20*), the subject of this presentation, are of considerable interest because their biological conversion efficiency is relatively high; also, the products utilized in the combustion reaction (H_2 and O_2) can be regenerated by electrolysis.

A number of observations indicate that hydrogen bacteria undergo autotrophic conversion according to the following equation:

$$6H_2+2O_2+CO_2\rightarrow(CH_2O)+5H_2O. \quad (1)$$

Electrolytic cleavage of 6 moles of H_2O would thus suffice to provide the products for the combustion reaction. Thus, the end result of biosynthesis plus electrolysis is the formation of 1 mole of CH_2O and 1 mole of O_2; this represents

TABLE I.—*Relationship Between Oxygen Consumed and Carbon Dioxide Assimilated, and Efficiency of Growth in a Number of Autotrophs*

	O_2/CO_2	Efficiency (percent)	Reference
Chemosynthesis:			
Thiobacillus thiooxidens [a]			
$S+1.5\ O_2+H_2O=H_2SO_4+104$	18	8.3	1, 2.
Nitrosomonas [a]			
$NH_4^+ +1.5\ O_2=NO_2^- +H_2O+2H^+ +84$ kcal	20–30	7.9	3, 4, 5, 6.
Nitrobacter [a]			
$NO_2^- +0.5\ O_2=NO_3^- +18$ kcal	30–50	5.9	7, 3, 4, 6.
Hydrogenomonas			
$H_2+0.5\ O_2=H_2O+57$ kcal	2	35	8, 9.
Algae (dark-reduction)			
$H_2+0.5\ O_2=H_2O+57$ kcal	2	35	10.
Photosynthesis:			
Algae [b]	----------	20	11, 12.
Chromatium spec	----------	10–20	13.

[a] The calculations of O_2/CO_2 quotients and energy efficiencies are based upon older data from the literature. It seems unlikely that the environmental conditions permitted maximum culture synthetic activity.
[b] The high efficiency is observed in light limited growth.

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the approximate caloric intake and oxygen required per hour by one man. Because the cleavage of 6 moles of H_2O requires approximately 600 kcal energy input (75 percent efficiency), the overall efficiency of energy conversion is in the order of 20 percent. This presentation will discuss in some detail the physiological requirements for batch and continuous cultures, the product composition and turn-over characteristics.

MINERAL NUTRITION

The nutritional requirements for *H. eutropha* were described by Repaske (ref. 14). Bartha (ref. 15) found a requirement for Ni and Eberhardt (ref. 16) stimulation of hydrogenase activity of Mn and Co. A more simplified medium was tested by Bongers (ref. 17). He found that rapid growth of *H. eutropha* and *H–20* could be obtained with a medium containing a urea or an ammonia-nitrogen source in the presence of magnesium, phosphate, and ferrous iron. Contaminations in the major salt ingredients made supplementation with trace elements unnecessary. This information on nutrient requirements for batch cultures is summarized in table II.

TABLE II.—*Optimal Growth Requirements of* Hydrogenomonas Eutropha

Density	10 grams (dry weight)/liter
Temperature	33° C–35° C.
Pressure	10^5 newton/meter^2.
Hydrogen	75%.
Oxygen	15%.
Carbon dioxide	10%.
$CO(NH_2)_2$	0.5 g/liter.
$MgSO_4 \cdot 7H_2O$	0.1 g/liter.
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.016 g/liter.
Phosphate buffer; pH	6.0–7.5.

When continuous-culture techniques, such as can be obtained with a turbidostat, are considered, the desired condition is a steady state with regard to cell concentration, concentration of nutrients, rate of cell multiplication, and all other properties of the cell suspension. Levels of nutrients should not change as they do in batch cultures. In order to keep the supposedly optimal concentration of the nutrients constant, the nutrients in the inflowing medium must be more highly concentrated than those in the suspending medium. This is because the inflowing medium is diluted by the suspending medium to an extent that depends on the consumption of the medium constituents by the cells. This relationship will be illustrated with substrate nitrogen as an example.

The simplest formulation for calculating the necessary concentration of nitrogen in the inflowing medium is:

$$N_m = N_s + N_p \qquad (2)$$

where N_m is the nitrogen concentration in the inflowing medium, N_s the concentration of the substrate nitrogen, and N_p the concentration of (working) protein (expressed as nitrogen) in the suspension. This formulation demonstrates that the nitrogen concentration of the inflowing medium (nitrogen feeding rate) depends on the protein concentration actually engaged in the reproduction process (nitrogen consumption rate) and on the level of substrate nitrogen to be maintained. Thus, a low consumption rate and a low substrate concentration require a low feeding rate. Conversely, a high consumption rate and a high concentration of substrate nitrogen require a high nitrogen feeding rate. A mismatched nitrogen feeding rate, and consequently a deviation from steady-state conditions, occurs if the actual nitrogen consumption rate deviates from the expected value. Deviations in the nitrogen consumption rate may occur if the population characteristics change with time, e.g., if the ratio of active metabolizing cells (working population) over resting cells (inactive population) changes in the course of an experiment.

Precautions such as those discussed for nitrogen must be considered for other elements (P, Mg, Fe, and trace elements) that are needed for cell reproduction. However, because these elements are tolerated at relatively high concentrations and have limited use, a substrate concentration approaching the concentrations in the inflowing medium should lead to steady-state conditions conducive to rapid cell reproduction. This assumption is not documented and merits experimental verification.

Another problem related to nitrogen metabolism and supply is the extremely rapid decomposition of urea, the primary nitrogen source in a closed environment. This conversion into NH_4^+ and CO_2 (and a concomitant upward pH shift) is to be expected with suspensions cultivated in the presence of relatively low concentrations of ammonia, according to König et al. (ref. 18). Under these conditions, cells seem to be formed, with a high urease activity. Conversely, in the presence of certain concentrations of ammonia, urease formation is depressed (see fig. 1).

We confirmed these observations—see figure 2 (a)—and in addition, found that the rate decomposition of urea was largely independent of the presence of an energy source; compare figure 2 (b) and (c). Apparently a given concentration of ammonia in the suspending medium may be necessary to prevent the build-up of high urease activity. A nitrogent supply technique responsive to the ammonia concentration may be needed to stabilize the conditions in the working suspension.

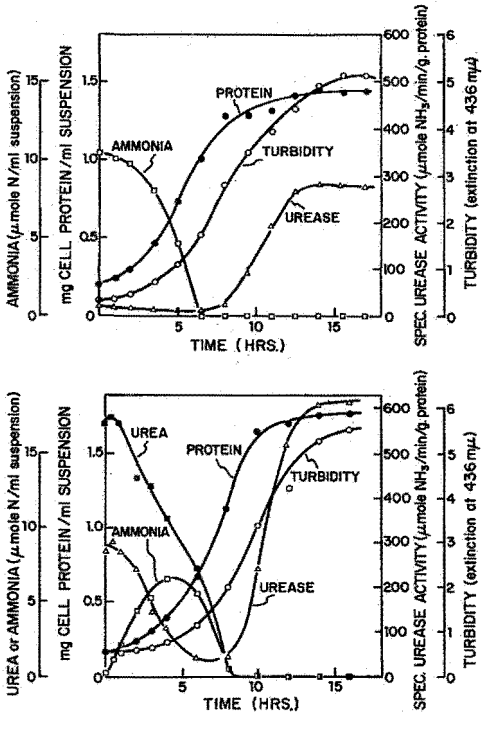

FIGURE 1.—Effect of ammonium ion concentration on urase activity in H–16 (according to König et al., ref. 18).

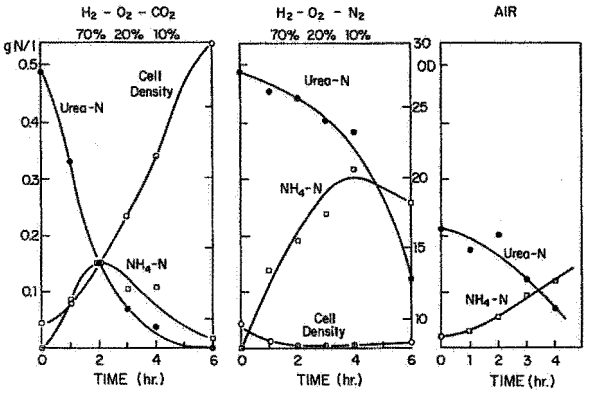

FIGURE 2.—Time course of accumulation of ammonium in the cell suspension of H–20 in the presence of urea. Gas phase: H_2, O_2, CO_2 (left); H_2, O_2, N_2 (center); and air (right).

SUPPLY OF GASEOUS SUBSTRATE

In the gaseous substrates that provide the suspension with energy and carbon dioxide, the hydrogen concentration could be varied widely (liquid phase tensions, 4 to 60 cm) with little effect on yield. Carbon-dioxide and oxygen concentrations were more critical. Carbon-dioxide concentrations up to 9 cm were tolerated but greater concentrations were harmful. The oxygen partial pressure of the growing suspension had a strong effect on rate of growth, energy-conversion efficiency, and metabolic activity with regard to product formation. At low oxygen concentrations (3.7 to 6 cm liquid phase tension), the rate of carbon-dioxide conversion was some 50 percent higher than at 15 cm. However, this relatively high rate could not be maintained. With prolonged incubation under these conditions, the rate of carbon-dioxide fixation declined rapidly, and intracellular lipid inclusions were formed. In these suspensions cell division declined rapidly. At high oxygen concentrations (15 to 20 percent) no lipid inclusions were formed, and the rates of cell division and carbon-dioxide fixation remained constant. The efficiencies of energy conversions were somewhat less than the values observed with low oxygen concentrations (see table III). This response to oxygen is illustrated in figure 3 and the optimal conditions for growth are summarized in table II.

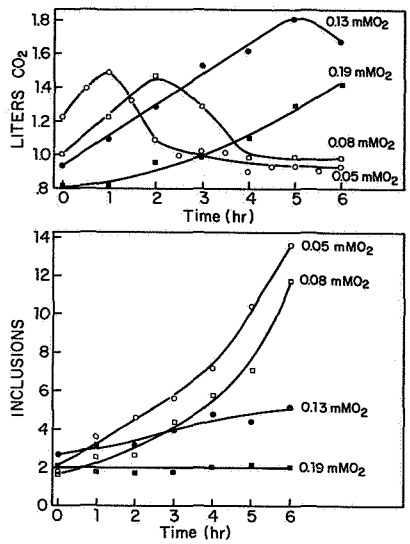

FIGURE 3.—Rate of CO_2 consumption (top) and formation of cellular (PBHB) inclusions (bottom) as a function of oxygen concentration at constant H_2 and CO_2 supply.

Also, nitrogen starvation alters the distribution of the products of carbon dioxide fixation (ref. 19); also, cell characteristics undergo changes like those obtained with prolonged incubation under low oxygen concentrations.

At present it is not precisely known what penalty this decrease in metabolic activity will exact on suspension volume and conversion efficiency. From the above discussion it is obvious, however, that cells starved for oxygen and nitrogen do not lend themselves to cultivation under steady-state conditions. The proportion of activity dividing cells in the total population would decrease, thus affecting the value N_p (cf. equation 2) and causing a transition to a new steady state. In order to cope with this problem, culture "staging" is proposed. This technique would involve cultivation in two phases occurring in two separate chambers; one culture chamber containing substrate nitrogen, the other not. At present there is no information on the adequacy of this approach, but it is expected to produce the best nitrogen economy in the closed environment.

TABLE III.—*Oxygen Supply and Energy Conversion. Gas Phase: 70-Percent H_2, 10-Percent CO_2 and O_2 as Indicated. Urea Medium; Temperature 35° C*

Percent O_2	5	10	15	20
O_2/CO_2	1.9±0.2	2.1±0.1	2.9±0.2	3.0±0.2

ENERGETICS

Hydrogen is used by hydrogen bacteria for reductive and energy-yielding purposes. Hydrogenase—an enzyme which mediates the use of hydrogen—catalyzes the reduction of some, yet unknown primary acceptor by hydrogen; this reduced acceptor then transfers electrons to other acceptors such as pyridine nucleotide, dyes, and (ultimately) oxygen. In this process, the two essential reactants required for CO_2 assimilation are generated: reduced pyridine nucleotide ($NADH_2$) and adenosine triphosphate (ATP).

In the combustion of 1 mole of H_2 by oxygen, 56 kcal are made available. In order to make the energy useful to the cell, it must be converted into a negotiable package of energy, i.e., ATP or its equivalent. The ATP thus generated can be utilized with $NADH_2$ for CO_2 reduction. In addition, ATP is spent for polymerization of primary products and for the performance of other functions, such as osmotic work and cell division. There is evidence that about 5 ATP are required for the complete synthesis of cell constituents.

Judging by the O_2/CO_2 ratios observed with autotrophic grown hydrogenomonads, the efficiency of ATP utilization must be rather low. Available evidence indicates (equation 1) that for the assimilation of 1 mole of CO_2, approximately 2 moles of oxygen are utilized for the combustion of hydrogen. If one assumes (ref. 20) that the intact cells have a P/O ratio equal to that observed with mitochondrial systems, then approximately 12 moles of ATP are required for the assimilation of 1 mole of CO_2. This is approximately twice the amount required for an efficient system.

At present, there is no evidence that the classical respiratory chain is operative in the whole cells. Evidence that an abbreviated chain is operative in cell-free preparations was described

TABLE IV.—*The Effect of Uncouplers and Inhibitors. Phosphorylative and Oxidative Activity with Hydrogen*

Substrate	Inhibitor or uncoupler	ΔP_i μmoles	ΔO_2 μatoms	P/O
	Uncouplers			
H_2	Control	3. 96	7. 95	0. 50
H_2	10^{-3}M DNP	1. 05	7. 05	0. 15
H_2	0.7×10^{-5}M CCCP	1. 17	6. 55	0. 18
H_2	10^{-3}M dicumarol	0	8. 0	0
	Inhibitors			
H_2	Control	4. 26	9. 2	0. 46
H_2	3×10^{-4}M CN^-	1. 64	3. 04	0. 48
H_2	10^{-4}M N_3^-	4. 20	9. 3	0. 45
H_2	160 mm (Hg) CO	4. 25	8. 6	0. 49
H_2	2 γ/ml Antimycin A	4. 05	8. 9	0. 45

by Bongers (ref. 17), who also studied the efficiency of ATP generation associated with the oxyhydrogen reaction. When oxidative and phosphorylative activities were compared in the presence and absence of specific inhibitors, effective coupling between electron transport and ATP generation was found at the first site of phosphorylation.

From the effects of CN^-, N_3^-, CO, and Antimycin A on both activities (see table IV), it was assumed that (at least with H_2 as electron donor) most of the phosphorylation occurs at a site not affected by these inhibitors, i.e., between hydrogen (or a low potential electron donor) and cytochrome *b*, and that this cytochrome is auto-oxidizable. This could be further demonstrated by spectrophotometric observations. As illustrated in figure 4, the cytochrome *b* absorption (curve 1, 562 mμ and 528 mμ), obtained by incubating cell-free extracts with H_2, disappeared when air was introduced in the presence of cyanide, while cytochrome *c* (curve 2, 552 mμ and 520 mμ) remained reduced. Because in the presence of cyanide cytochrome *c* is reduced even in air, reduced cytochrome *b* is the only difference between the hydrogen-flushed sample cuvette and the air-equilibrated reference cuvette (see fig. 4, curve 3). Again, substitution of H_2 by air yielded reoxidation of cytochrome *b* (fig. 4, curve 4). This observation illustrates that under certain conditions cytochrome *b* can function as a terminal oxidase; moreover, it explains the inability of terminal oxidase inhibitors to inhibit electron flow, and it verifies the assumption that with H_2 as electron donor the bulk of phosphorylation occurs at a lower potential than cytochrome *b*.

An attempt was also made to assess the ATP requirement for total cell synthesis by finding the efficiency of CO_2 fixation (O_2/CO_2) in the presence of specific inhibitors. In addition, reduction and oxidation of cytochromes *b* and *c* was spectrophotometrically determined in whole cells in the presence and absence of cyanide. The number of inhibitors that can be used with intact cells is limited. Actually, only Site III (between cytochrome *c* and O_2) can be effectively inhibited by either cyanide, azide, or carbon monoxide. No effective inhibitors are known that block the electron transfer at Site II (between cytochrome *b* and cytochrome *c*) in intact cells. However, it was observed with cell-free extracts that no reaction between reduced cytochrome *c* and oxygen occurred in the presence of 10^{-4}M cyanide. A similar inhibition can thus be assumed to occur in intact cells. It is therefore unlikely that in the presence of terminal oxidase inhibitors Site II will contribute substantially in the overall ATP formation.

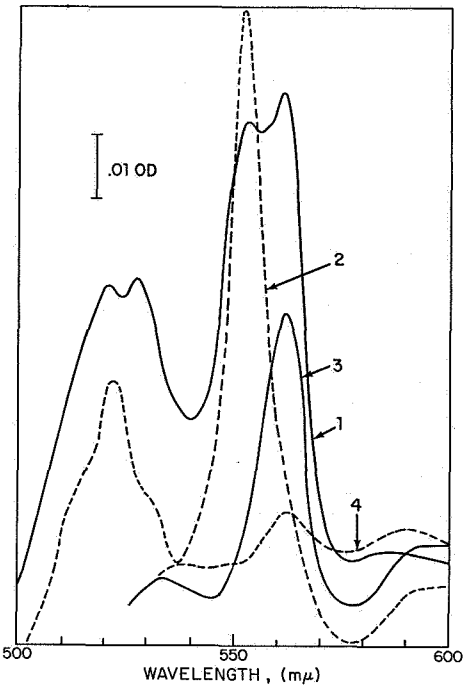

FIGURE 4.—Oxidation of reduced cytochrome b by oxygen. Sample (Thunberg) cuvette containing 2-ml extract (10-mg protein per ml) and cyanide in the side arm, was gassed with hydrogen. Reference cuvette contained 2-ml extract in air. After an incubation of 5 minutes at room temperature, spectrum 1 was obtained. Subsequently, cyanide was tipped (final conc. 10^{-4}M), the hydrogen in the sample cuvette replaced by air and spectrum 2 was obtained. Spectra 3 and 4 were obtained with 10^{-4}M CN^- in both reference and sample cuvette. Spectrum 3 was measured after flushing the sample with hydrogen and the reference with air. Spectrum 4 was obtained subsequently after slushing the sample cuvette with air. Measurements were made at room temperature in Cary model 15.

The three inhibitors mentioned above not only inhibit electron transfer via cytochrome *a* to oxygen, but also react with other metalloenzymes (e.g., hydrogenase, catalase, aldolase), but cytochrome oxidase has probably the greatest sensitivity known. Due to these side effects of cytochrome oxidase inhibitors, a considerable decrease in the rate of growth can be expected. Actually, it is not the rate of growth that is of interest but their effect on conversion efficiency.

Table V lists a number of observations concerning conversion efficiency in the presence and absence of terminal oxidase inhibitors. The conversion efficiencies are expressed as the O_2/CO_2 ratio. This ratio expresses the number of hydrogen oxidations required for the assimilation of one carbon dioxide. Under normal conditions of cultivation, a value of 2 is observed. It can be seen from table V that neither cyanide, axide, nor carbon monoxide substantially changes this value. Even a large number of observations shows no substantial increase in the O_2/CO_2 ratio; this proves that in the uninhibited system, also, Site I satisfies the necessary ATP requirement. The CO_2 assimilation observed in the presence of H_2 and O_2 depends on ATP formation, generated by oxidative phosphorylation; this is evident from the fact that little C^{14} incorporation is observed with cells incubated in the presence of an uncoupler (CCCP) of oxidative phosphorylation. Also, the cells incubated in the presence of air or an air-CO mixture showed little CO_2-fixing activity. The CO_2 assimilation observed is thus a net synthesis that does not depend on substrate level phosphorylation.

Spectrophotometric observations on intact cells, illustrated in figure 5 (curves 1 and 2)

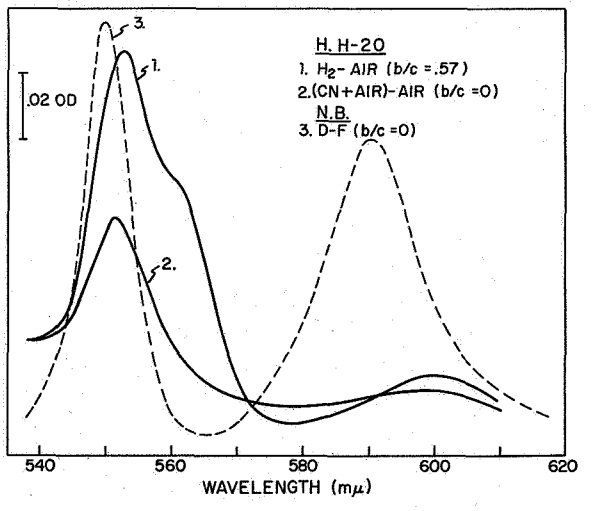

FIGURE 5.—Curve 1: Absorption spectrum of intact H–20 cells (hydrogen reduced minus air oxidized); absorption maxima at 552 mμ (cytochrome c) and 562 m (cytochrome b). Curve 2: Absorption spectrum of intact cells (CN^- difference spectrum); absorption maximum at 552 mμ (cytochrome c), cytochrome b is oxidized. Curve 3: Absorption spectrum of intact Nitrobacter agilis cells (dithionite minus ferrocyanide); absorption maximum 550 mμ (cytochrome c) and 590 mμ (cytochrome a).

TABLE V.—*The Effects of Inhibitors on Conversion Efficiency*

Series	Incubation (min)	Condition of incubation: Gas phase (percent) H_2	O_2	CO_2	N_2	Inhibitor	CO_2 fixation (μmoles/hr)	O_2 consumption (μmoles/hr)	O_2/CO_2
I	80	60	15	5	20	Control	32	63	1. 95
	80	60	15	5		20% CO	17	43	2. 50
	90	60	15	5	20	Control	41	95	2. 30
	90	60	15	5		20% CO	22	45	2. 05
II	0	70	20	10		Control	3. 8	8. 0	2. 1
	10	70	20	10		10^{-4}M NaN_3	4. 3	8. 7	2. 0
	35	70	20	10		10^{-4}M NaN_3	3. 7	8. 8	2. 4
	60	70	20	10		10^{-4}M NaN_3	2. 6	8. 8	3. 4
	120	70	20	10		10^{-4}M NaN_3	1. 1	6. 8	6. 2
	60	70	20	10		Control	4. 3	8. 2	1. 9
							Gas uptake (μl/flask)	CO_2 fixation (CPM)	Counts/μl
III	10	55	5	40		Control	370	54 000	146
	10	55	5			40% CO	127	23 400	184
	10 [a]	55	5	40		Control	131	11 500	88
	10 [a]	55	5			40% CO	36	4 290	119
	10	80	20			Control	152	16 100	106
	10	80	20			10^{-4}M NaN_3	150	15 700	104
	10	80	20			10^{-3}M NaN_3	124	9 700	78
	14	80	20			Control	226	23 000	102
	14	80	20			10^{-4} CN^-	86	9 900	115
	14 [a]	80	20			Control	203	21 500	106
	14 [a]	80	20			10^{-4} CN^-	77	6 600	86
	10	80	20			Control	165	16 400	100
	10	80	20			5×10^{-5}M CCCP	130	2 500	19

[a] Suspension aerated (in air) for 1 hour before incubation.

gave similar indications as cell-free extracts (see fig. 4) with regard to the role of cytochrome *b*. In the presence of CN only the absorption due to cytochrome *c* (fig. 4, curve 2) is observed, while cytochrome *b* is completely oxidized. Kinetic studies have shown that this oxidation of cytochrome *b* by oxygen occurs at relatively low oxygen concentration, indicating a high affinity of cytochrome *b* for oxygen. Whatever the precise nature of the processes involved in this oxidation, the observation clearly complements the experiments described above and reinforces the conclusion that a cytochrome *b*-oxidase is operative in the intact cells. In order to supply energy for CO_2-fixation, the cell apparently depends upon an abbreviated electron transport chain ($H_2 \rightarrow Q \rightarrow b \rightarrow O_2$). See figure 6.

In all cell-free extracts as well as in intact cells examined spectrophotometrically, there was no convincing evidence of the presence of cytochrome oxidase. (Figure 5, curve 3 shows cytochrome *c* and *a* in *Nitrobacter agilis*.) It is not known whether this behavior of the

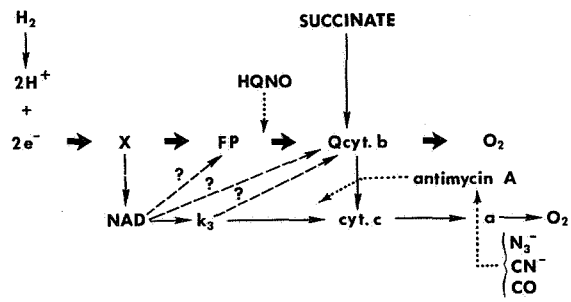

FIGURE 6.—Electron transport scheme for cell-free extracts of Hydrogenomonas sp.

cytochrome *c*-oxidase is a characteristic of all hydrogenomonads or an adaptive response to cultural conditions. It may well be that the cytochrome *c*-oxidase content strongly depends on the oxygen concentration at which the cells are grown. The relatively high concentrations of oxygen normally used for the cultivation may well be responsible for the depression of oxidase development. Consequently, electron transfer via cytochrome *c* to oxygen may become slow, and a changeover to a cytochrome *b* oxidase may be the result. This changeover in pathway would, as mentioned earlier, decrease the efficiency of the oxyhydrogen reaction. It is conceivable that the cell, utilizing the abbreviated pathway, can make energy available rapidly enough to satisfy the requirements.

STEADY-STATE TURNOVER

Not enough observations are available at present to give a meaningful evaluation of the rate of growth under steady-state conditions. Of the test experiments carried out to date in the device illustrated in figure 7, only a few runs of adequate duration have been made at constant conditions, since variables have been changed often in attempts to produce optimal growth conditions. With these qualifications in mind, the data discussed here must be considered.

The cultures were initiated at a cell density of approximately 4 grams of dry weight per liter of resuspending freshly harvested cells in a fresh medium. No attempts were made to obtain high cell concentrations in the autoculture by starting out with a small inoculum. The gas concentrations maintained during the test runs were as follows: 12-percent oxygen, approximately 9-percent CO_2 and H_2 as balance. The working pressure was 3.5 psig, and the temperature of the suspension 30° to 32° C.

In figure 8 the K-values (dimension of $K = hr^{-1}$) of two runs are shown. All K-values observed during the 4- to 5-day runs fell between the high and the low values shown here (fig. 8), and confirm the observations made by Foster et al. (ref. 22).

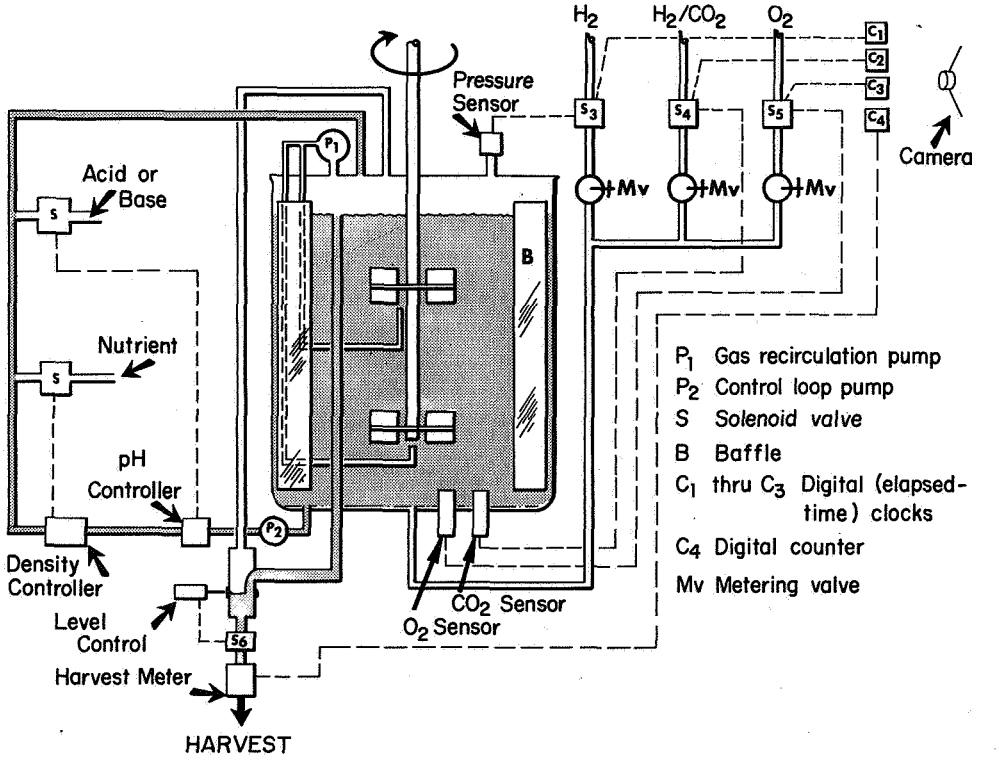

FIGURE 7.—Autoculture unit; total volume 5 liters; working volume 4 liters. Configuration according to Finn (ref. 21); design adapted from Foster et al. (ref. 22).

The rate of CO_2 consumption varied from 0.7 liter CO_2 to 0.9 liter of CO_2 taken up per liter of working suspension per hour. Also, considerable variation in the rate of O_2 consumption was noted. Consequently, the efficiency of energy conversion (O_2/CO_2) varied widely. The best value was 2, which is equal to the best values found with batch cultures. The highest O_2/CO_2 value was on the order of 4, representing the lowest conversion efficiency. If one assumes that the conditions can be manipulated to produce a steady-state rate of $K=0.20$, extrapolation of the data points to a suspension-volume requirement of approximately 20 to 30 liters per man.

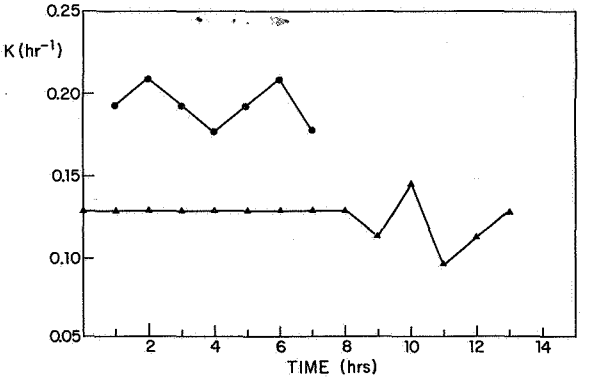

FIGURE 8.—Specific rate of cell production during steady-state operation (density of working suspension approximately 5 grams dry weight per liter).

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COMMENTS

Dr. REPASKE. It seems that you might be able to control your urease activity by feeding both ammonia (to maintain the urease) and urea simultaneously, if you can control the level of ammonia accurately.

Dr. BONGERS. If you give a mixture of urea and ammonia you are in business. But, then you have to control also the ammonia in the suspension all of the time and maintain its constant. I believe it can be done without too much difficulty.

Dr. REPASKE. I am interested, too, in your oxygen-CO_2 ratios in the presence of carbon monoxide (CO). We find that carbon monoxide inhibits hydrogenase. I see that your oxygen-CO_2 ratios may remain the same. If I recall the figures correctly, your net oxygen uptake is lower; I thought it was the same.

Dr. BONGERS. No, much lower; we have done the experiment at different levels. In general, an oxidase is very sensitive to carbon monoxide or cyanide, very sensitive relative to hydrogenase. You can give a very low concentration of carbon monoxide and just not inhibit hydrogenase. If there is inhibition, it is very limited. We have done the experiments at very low CO_2 concentrations and at very high CO_2 concentrations, and essentially the O_2/CO_2 ratio does not go down. I have no idea how we can explain this.

Dr. REPASKE. Endogenous?

Dr. BONGERS. Endogenous? If you look at the same cells under conditions where you substitute hydrogen ^{14}C for nitrogen, the amount of ^{14}C fixed is about

zero. If you take out only the oxygen and give it hydrogen, the amount of ^{14}C fixed is very limited. It is definitely not endogenous. You can completely stop the C-fixation with ATP, but not as well with DNP or with arsenate. Of course, they are apparently both incapacitated by hydrogen.

Dr. HUMPHREY. Is it possible that your low efficiency might be due to an equipment anomaly? I notice in the way you operate your turbidostat that you are sucking the material from the surface. The lipid-rich cells will tend to go into the foam because of the lipolytic action. They will tend to concentrate in the foam; you are sucking those off. And, are not the lipid-rich cells the ones that are more efficient in fixing your CO_2?

Dr. BONGERS. Any cells which go off have to go to the bottom. It would not seem that this can explain the discrepancy.

Dr. MCFADDEN. It would seem that the problem in these chemostat experiments might be a drift of the composition of the recirculated gases. Do you encounter this problem? If the oxygen-CO_2 ratio is changing, this could result in a change in composition of the gases that are to be recirculated. I think that this might be an important problem for the long term. Don't you depend on more or less matching the input gas composition with that used by the cells?

Dr. BONGERS. Not essentially. What you do is to place your sensors in your liquid phase. When you measure this way, you do not care a bit what is above.

Dr. MCFADDEN. But you are recirculating these gases.

Dr. BONGERS. Yes.

Dr. MCFADDEN. So, it seems to me you are changing the composition with time.

Dr. BONGERS. No, you are not changing that much with time, because your cell concentration stays constant. Only if the consumption should change drastically (let us say it would take up much more oxygen at one time than another) your composition should change.

Dr. MCFADDEN. Right. And it does change as much as twofold.

Dr. BONGERS. I do not know how much it can be.

Dr. DECICCO. I do not think you are getting recirculation in the way you estimate. This is a one-way feed. You do not recirculate your complete mixture. Is that right?

Dr. BONGERS. You recirculate some of it.

Dr. FOSTER. He is recirculating some of the gases. But, you are also feeding the gases in from the bottom. Our experience is that you get equilibrium within less than a minute, at least.

Dr. BONGERS. Yes, easily. I believe there is equilibrium at all times.

Dr. REPASKE. It may cost us two sites of phosphorylation, because of our methods of measuring those two sites.

Dr. BONGERS. I believe it is due to our conditions. If you could cultivate them in a very low oxygen concentrate, you definitely would develop a cytochrome oxidase.

Dr. DECICCO. If I remember correctly—maybe Dr. Repaske would know better than I—I do not believe a bacterial system has ever been found that gives you a better P-to-O ratio than 1-point-something. Is that not true? I know a lot of them have been checked. But, in general, they are very low. You cannot get anywhere near that which you would get with a mammalian system.

Dr. REPASKE. That is generally true. But in *Microbacterium phliae* it has approached 3.

Biochemistry and Physiology of Hydrogen Bacteria: Biochemical Differences in Hetero- and Autotrophs

BRUCE A. MCFADDEN
Washington State University
Pullman, Washington

Use of the hydrogen bacteria as a component in bioregenerative life-support systems during space flight will depend on a number of factors. One will be a thorough understanding of their metabolism. Several species undergo readily reversible transition from autotrophism to heterotrophism. We have examined the carbon metabolism of one of these species, *Hydrogenomonas facilis*, in an effort to define the differences between these two modes of growth in molecular terms.

In 1963 (ref. 1) we discovered that cells grown, washed, and presented with growth substrate under growth conditions coupled ribose oxidation with $^{14}CO_2$ fixation very efficiently. These studies were extended to investigate the mechanism of $^{14}CO_2$ fixation in very short fixation times (ref. 2). Fixation of radiocarbon into glutamate that depends on the oxidation of ribose was observed. Total incorporation was markedly inhibited by uncouplers of oxidative phosphorylation. Thus characteristics of the observed incorporation of 14-carbon into glutamate suggested that it might be important in the biosynthesis of glutamate. An interesting fact is that intermediates of the tricarboxylic acid (TCA) cycle were not detectably labeled. Exhaustive efforts to identify the very early products of carbon-dioxide fixation were unsuccessful. However, the relatively early labeling of glutamate and of phosphoglycerate (3–PGA) and metabolically related C_3-compounds afforded a means by which the mechanism of CO_2 fixation could be examined. Accordingly, glutamate and 3–PGA have been isolated and degraded by standard chemical, enzymatic, and microbiological techniques. In addition, after a separate fixation experiment, pyruvate has been isolated as the 2,4–dinitrophenylhydrazone, the derivative hydrogenolyzed to alanine and the alanine decarboxylated with chloramine T. Results are summarized in table I.

TABLE I.—*Labeling Patterns in Purified Intermediates of $^{14}CO_2$ Fixation During Ribose Oxidation*

Compound	Percent label fixed			Distribution (percent)		
	6 sec	10 sec [a]	12 sec	6 sec	10 sec [a]	12 sec
Glutamate	3.9	*ca.* 4	3.8			
C_1				90	80	48
C_5				10	20	51
3–PGA	5.6		9.7			
C_1				99		99
C_2				0		0
C_3				0		1
Pyruvate		*ca.* 2				
C_1					89	

[a] Both of these 10-second experiments were conducted separately and were also distinct from the 6- and 12-second experiments, which were conducted simultaneously with cells of the same origin. Consequently data are not strictly comparable except for the 6- and 12-second fixations.

The kinetics of labeling of 3–PGA during autotrophic fixation by *H. facilis* suggest that 3–PGA is the first stable product of CO_2 fixation (ref. 3) and additional data establish that the Calvin cycle is the major fixation path (refs. 3 and 4). The data in table I suggest that 3–PGA arises from function of the Calvin cycle, which operates at a suppressed rate compared with that during autotrophic fixation. A similar conclusion was reached by Hirsch et al., who studied products after a single fixation time by *Hydrogenomonas H16* oxidizing succinate (ref. 5). The results (table I) also suggest that pyruvate arises from 3–PGA as the result of catalysis by phosphoglyceromutase, enolase, and pyruvate. Of particular interest to us were the labeling patterns obtained for glutamate (table I). Incorporation of $^{14}CO_2$ into C_1 was clearly consistent with the TCA cycle; yet, as mentioned before, intermediates of the cycle were not detectably labeled. We therefore examined the possibility that enzymes of the TCA cycle were repressed by growth of *H. facilis* on ribose by comparing levels of these enzymes in ribose- and yeast extract-grown cells. Table II presents data for extracts prepared by sonic disruption (SO) or with the French pressure cell (FPC).

TABLE II.—*Enzyme Assays*

Enzyme	Assayed at (mu)	Cells ruptured by	Percentage of "C" activity in "S"		Specific activity in "C" mμmoles/min/mg protein	
			Ribose-grown	YE-grown	Ribose-grown	YE-grown
NADPH oxidase	340	FPC+SO	------	------	barely detectable	
NADH oxidase	340	FPC	28	27	21	52
Pyruvate dehydrogenases	418	SO	80	none	19	2. 3
Citrate condensing enzyme	232	FPC	86	94	226	323
Aconitase	240	SO	46	83	30	31
Isocitrate dehydrogenase ($NADP^+$)	340	SO	93	96	148	71
Isocitrate dehydrogenase (NAD^+)	340	SO	------	------	undetectable	
α-ketoglutarate dehydrogenase	418	SO	none	none	2. 2	1. 5
Succinate dehydrogenase	600	FPC	none	25	323	610
Fumarase	240	SO	95	81	341	243
Malate dehydrogenase (NAD^+)	340	FPC	106	110	4360	3530
Malate dehydrogenase ($NADP^+$)	340	FPC	113	105	72	206
Isocitrate lyase	520	SO	------	[a] 100	[b] 16	[a] 23
Malate synthase	412	SO	------	[a] 100	[b] 102	[a] 65
Lactate dehydrogenase (NAD^+ and $NADP^+$)	340	FPC+SO	------	------	undetectable	

[a] See reference 6. Specific activity is for fraction S.
[b] Specific activity is for fraction S.

Specific enzyme activities are reported only for fraction "C," which was the supernatant from centrifugation at 1500g after the disruption of cells. However, the percentage of "C" activity that was recovered in the supernatant, "S," after recentrifugation of "C" at 144 000g was also determined and is reported. As is evident, all enzymes of the TCA cycle are detectable at similar levels in ribose- and yeast extract-cultured cells. This is true also of the metabolically related enzymes, isocitrate lyase and malate synthase. NADH oxidase, aconitase and the dehyrogenases for pyruvate, α-ketoglutarate and succinate appear to originate in particles. These data (table II) and the recent results of Homann (unpublished observation) who has found rapid oxidation of succinate, fumarate, malate, pyruvate, oxalacetate and α-ketoglutarate by ribose-grown cells, leave little doubt about the function of the TCA cycle in *H. facilis*. Trüper (ref. 7) has recently come to a similar conclusion on the basis of enzyme studies of *Hydrogenomonas* H16G+. It is interesting that, with the latter organism, autotrophic cul-

ture significantly suppresses levels of enzymes of the TCA cycle.

Our findings, nevertheless, left open the possibility that ribose-grown *H. facilis* did not possess one or more of the usual carboxylation enzymes that afford conversion of C_3-compounds to intermediates of the TCA cycle. This possibility was examined next and the observations are summarized in table III.

In mixture 1, heavy meromyosin was employed to generate GDP from GTP. The data suggest that phosphoenolpyruvate (PEP) carboxykinase, pyruvate carboxylase, malic enzyme (NADH- and NADPH-linked), and PEP carboxylase that utilizes inorganic phosphate as a phosphate acceptor play only a minor role in carboxylation. Instead, an acetyl CoA-stimulated PEP carboxylase is the chief catalyst of carboxylation. Heretofore it has been reported only in the enteric bacteria (refs. 8 and 9). After further study, the specific activity of the acetyl CoA-stimulated PEP carboxylase in ribose-grown *H. facilis* was estimated to be 0.60 mμmole CO_2 fixed/mm/mg protein at 30° C. Although low calculations establish that this level is adequate to account for the observed rate of C_1 labeling of glutamate, if it is assumed that carboxylation is followed by product turnover through the TCA cycle leading to α-ketoglutarate and subsequently to the amino acid.

TABLE III.—*Carboxylation of Pyruvate and Phosphoenolpyruvate (PEP) by $H^{14}CO_3^-$*

Major components in incubation mixture [a]	$^{14}CO_2$ incorporation, cts/5 min	
	After ribose growth	After YE growth
PEP, IDP, ADP, GTP, heavy meromyosin (HMM)	1271	3052
IDP, ADP, GTP, HMM	705	1613
PEP, IDP, ADP	1233	2000
Pyruvate, NADH	630	2093
NADH	700	2021
Pyruvate, ATP, biotin	895	1997
ATP, biotin	1080	1526
Pyruvate, ATP	986	2100
Pyruvate, NADPH	1876	3449
NADPH	1562	2623
Pyruvate	703	1902
PEP, acetyl-CoA	33800	----------
acetyl-CoA	3210	----------
PEP	8530	10650
PEP, inorganic phosphate	8100	7838
inorganic phosphate	2410	1964

[a] Cells were disrupted with a French pressure cell in the presence of 4 *mM* GSH under the usual conditions. The supernatant from 20 000g (20 min; 2° C) was then used.

These observations, then, raise a question about the failure in earlier experiments (fig. 2) to detect labeled TCA cycle intermediates arising from $^{14}CO_2$ fixation during ribose oxidation. Perhaps in those experiments this was only due to reflected pool sizes or the loss of these intermediates during volume reduction of the acidified ethanolic extracts.

Of major interest in the present work is the rapid labeling of the γ-carboxyl group of glutamate. One widely overlooked reaction that could partially account for this would involve catalysis by isocitrate lyase, which was shown to be

present in ribose-grown cells (table II). Thus, isocitrate derived from ^{14}C–1,4-oxalacetate would be cleaved to succinate labeled in a single carboxyl carbon. Reversal of the reaction would result in incorporation of ^{14}C into the γ-carboxylate group of isocitrate and eventual labeling of C_5 of glutamate. The ^{14}C–1,4-oxalacetate would arise from carboxylation of carboxyl-labeled PEP, in turn derived from labeled phosphoglycerate. The amount of label in each of the carboxyls of oxalacetate would approach equality as a consequence of turnover through catalysis by malate dehydrogenase and fumarase. Under the latter conditions, the specific radioactivity of C_1 of glutamate would be twice that of C_5 in the limit. In the present work, the extent of labeling of C_1 and C_5 was about equal in the longest fixation time.

There are other paths that might contribute to synthesis of ^{14}C–1, 5–glutamate (fig. 1). The relative rates of labeling of C_5 and C_1 of glutamate by path two would depend critically upon the rate of the aforementioned equilibration of label between the carboxyls of oxalacetate and the specific radioactivity of each of these two glutamate carbons would be equal in that limit. Participation of path two in glutamate biosynthesis by extracts of *Acetobacter suboxydans* can be tentatively inferred from the date of Sekizawa et al. (ref. 10), although very long incubations were conducted. If path two functions in *H. facilis*, this organism should be an excellent source of the enzymes. A possibility that could account for the observed C_5-labeling of glutamate would first involve conversion of oxalacetate–1,4–^{14}C to pyruvate–1–^{14}C. Condensation of the latter compound with acetyl CoA would give rise to citramalate, which might then lead to glutamate–5–^{14}C (ref. 11). Another possibility that would account for C_5-labeling would involve catalysis in path one by a citrate synthase of abnormal stereospecificity, as found recently by Gottschalk and Barker for *Clostridium kluyveri* (ref. 12). Further work on the biosynthesis of glutamate by *H. facilis* is anticipated in our laboratory.

Our data with fixation of $^{14}CO_2$ by intact cells oxidizing ribose (table I) suggested that the Calvin cycle functioned at a reduced rate. For several years now we have been conducting parallel studies at the subcellular level to discover metabolic loci in the Calvin cycle that were sensitive to repression or feedback inhibition during heterotrophic growth. At the outset, we postulated that a complement of enzymes that functions uniquely in the reductive pentose phosphate pathway (Calvin cycle) for CO_2 fixation is synthesized as part of the adaptation to autotrophism. Conversely, we presumed that synthesis of the same complement would be suppressed during heterotrophic growth. Resultant levels of these enzymes might then control the relative amounts of CO_2 fixed by

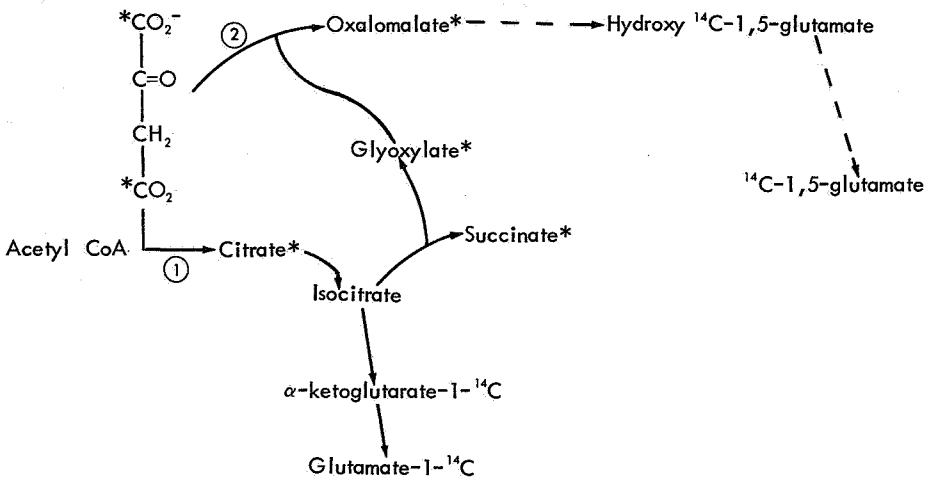

FIGURE 1.—Sample of glutamate synthesis path.

various paths during heterotrophic and autotrophic growth.

Two enzymes that ostensibly function only in the Calvin cycle are ribulosediphosphate (RuDP) carboxylase and phosphoribulokinase (Ru5–P kinase). About 4 years ago we discovered a cell-free ATP-, NADH-dependent CO_2 fixation (ref. 13). Because of its properties, we felt that fixation was occurring via the Calvin cycle. The prospect of correlating the level of this fixation with the level of one or more catalysts in the Calvin cycle in probing for control points in CO_2 fixation was quite attractive. Therefore, we thoroughly characterized the fixation. Figure 2 shows the dependence of fixation upon NADH and ATP. NADPH was 3 percent as effective as NADH as a reductant. Table IV illustrates the dependence of fixation upon HCO_3^- (provided at constant specific radioactivity) and Mg^{+2}. Figure 3 presents data on the time course of ATP-, NADH-dependent CO_2 fixation. It can be estimated that only cyclic fixation can accommodate the level of CO_2 fixation observed. Noncyclic fixation reactions would have required extraction from the cells of CO_2 acceptor(s) that had been present in the cell at a total concentration of 10 to 15 moles per liter. Incorporation by exchange is rendered unlikely by the marked dependence upon ATP and NADH and the prolonged constant rate of incorporation. Thus, all properties of the fixation suggested that it occurred via the reductive, energy-requiring pentose phosphate cycle.

In parallel work, optimal conditions for the measurement of RuDP carboxylase were established and the major product of RuDP-dependent $^{14}CO_2$ fixation was proved to be ^{14}C–phosphoglycerate (ref. 10). However, assay of Ru5–P kinase proved much more difficult because published methods for preparation of the substrate failed. Extracts of *H. facilis* contained ribose phosphate isomerase. Therefore, we decided to generate D-ribulose 5-phosphate (Ru5–P) from added D-ribose 5-phosphate (R5–P). After incubation of extract with R5–P, the kinase reaction was initiated with ATP. Simultaneously, $H^{14}CO_3^-$ and exogenous spinach RuDP carboxylase were added. In this manner, limiting kinase was coupled with exogenous carboxylase and it was presumed that the rate of $^{14}CO_2$ incorporation into acid stable product would equal the rate of the Ru5–P kinase-catalyzed reaction. In all studies, three different lengths (10, 20, and 30 minutes) of preincubation of extract with R5–P were conducted prior to the addition of ATP, $H^{14}CO_3^-$ and exogenous carboxylase. It was assumed that if saturating levels of Ru5–P had accumulated in the preincubation periods, subsequent incubation for 5 minutes to monitor the kinase would yield kinase levels independent of the preincubation periods. This proved to be true in most cases; exceptions will be noted in summarizing the data.

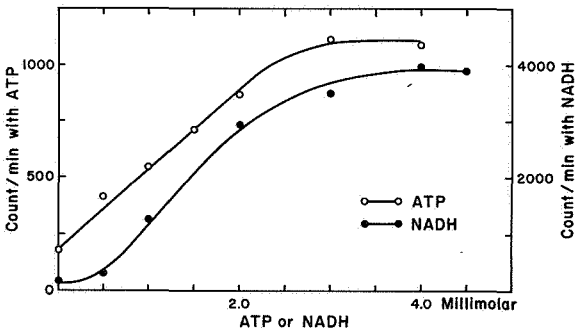

FIGURE 2.—Dependence of $^{14}CO_2$ fixation upon ATP and NADH.

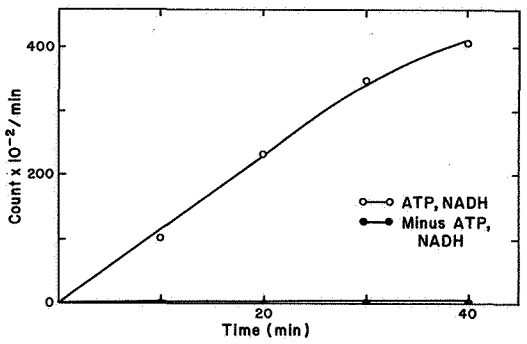

FIGURE 3.—The kinetics of ATP-, NADH-dependent $^{14}CO_2$ fixation.

Table V displays data which establish that the two levels (13.3 and 20 milliunits) of exogenous spinach RuDP carboxylase employed in all assays were indeed excessive. Figure 4 illustrates the dependence of Ru5–P kinase on R5–P and ATP. Magnesium ion at 20 mM was used in these experiments and the apparent

TABLE IV.—*The Effect of $MgCl_2$ and $H^{14}CO_3$ on Reductive ATP-Dependent $^{14}CO_2$ Fixation*

$MgCl_2$ varied (*mM*)	Complete (count/min)	Complete minus ATP and NADH (count/min)	$H^{14}CO_3$ varied [a] (*mM*)	Complete (count/min)	Complete minus ATP and NADH (count/min)
1	1 000	886	10	45 076	756
11	14 024	1148	20	96 564	662
101	15 845	1156	25	110 454	680
			30	102 808	700

[a] Provided at constant specific radioactivity.

TABLE V.—*The Amount of RuDP Carboxylase Required for the Ru5–P Kinase Assay*

Milliunits of RuDP carboxylase added	Ru5-P kinase activity	
	Complete (count/min)	Complete minus ATP and R5–P (count/min)
20	2400	70
13. 3	2482	67
10	1876	76
6. 7	1479	64
None	624	66

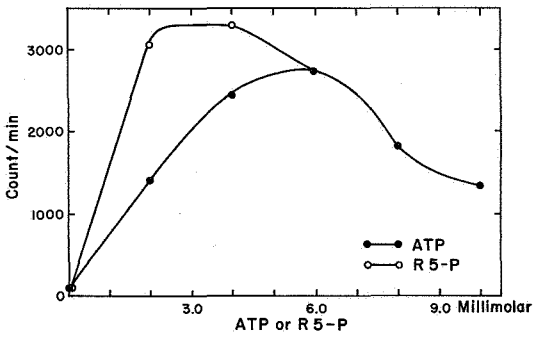

FIGURE 4.—Dependence of Ru5–P kinase activity on R5–P and ATP. Preincubation of 10 minutes occurred before addition of $H^{14}CO_3^-$.

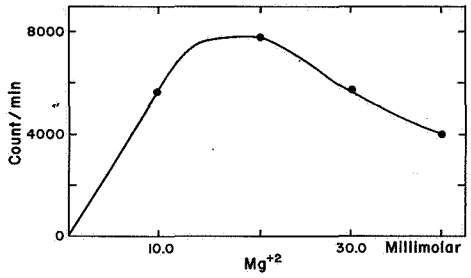

FIGURE 5.—The effect of $MgCl_2$ upon Ru5–P kinase.

inhibition especially at higher ATP concentrations, may only have been due to desaturation of one of the catalysts with respect to Mg^{+2}. Figure 5 shows the dependence of the kinase upon $[Mg^{+2}]$. Figure 6 shows radioautographic evidence that the only acid-stable radioactive product in the incubation mixture designed to measure Ru5–P kinase is phosphoglycerate, as would be expected.

With the establishment of apparently optimal conditions for the assay of ATP-, NADH-dependent CO_2 fixation, RuDP carboxylase and Ru5–P kinase, the specific activities for these processes were determined in regions of first-order dependence upon protein concentration. The data are summarized in table VI.

As evident, RuDP carboxylase and Ru5–P kinase are apparently not coordinately regulated. On the other hand, inspection of the data in table VI reveals that the specific activity of RuDP carboxylase approximately parallels that of reductive ATP-dependent CO_2 fixation. If these activities are coordinately regulated, the ratio of their specific activities should be constant over the wide range of growth substrates employed. Division of data in column b by the corresponding data in column e (or d) gives a mean ratio of 9.07. Mean deviation of individual ratios is 42 percent, revealing remarkable constancy considering the complexity of one of the processes, i.e., ATP-, NADH-dependent CO_2 fixation. On the other hand, the mean deviation of individual ratios of c/e (or d) is 80 percent. Thus, the kinase activity does not parallel CO_2 fixation nearly as closely as does that of RuDP carboxylase.

The observed suppression of specific activities of kinase and carboxylase after various

TABLE VI.—*The Specific Activities* [a] *of RuDP Carboxylase, Ru5-P Kinase, and ATP-, NADH-Dependent CO_2 Fixation*

Cultured	RuDP carboxylase (milliμmoles CO_2 fixed/min/mg protein)	Ru5-P kinase (milliμmoles RuDP formed/min/mg protein)	ATP-, NADH-dependent CO_2 fixation (milliμmoles CO_2 fixed/min/mg protein)	
	b*	c*	d*	e*
Autotrophically	67. 3	24. 7[b] (19. 7)	4. 75	(4. 54)
On fructose	51. 1	24. 0[b] (22. 3)	4. 83	
On glucose	51. 4	23. 2[b] (21. 4)	1. 65	(3. 10)
On ribose	24. 6	15. 2[b] (12. 2)	2. 07	(2. 87)
On glutamate	2. 05	12. 8	0. 85	
On lactate	10. 2	12. 9	1. 73	(1. 51)
On succinate	2. 25	13. 4	0. 24	
On acetate	1. 4	2. 8[b] (2. 43)	0. 41	

[a] All the data are corrected for results with controls which lacked substrate(s).
[b] These values are calculated from the 10-min preincubation experiment while all others including those in parenthesis represent the average of experiments involving 10-, 20-, and 30-minute preincubations.
*The three experiments b, c, and e were conducted on the same extracts; in d and e the experimental conditions were identical except that 12 *mM* and 20 *mM* $MgCl_2$, respectively, were used.

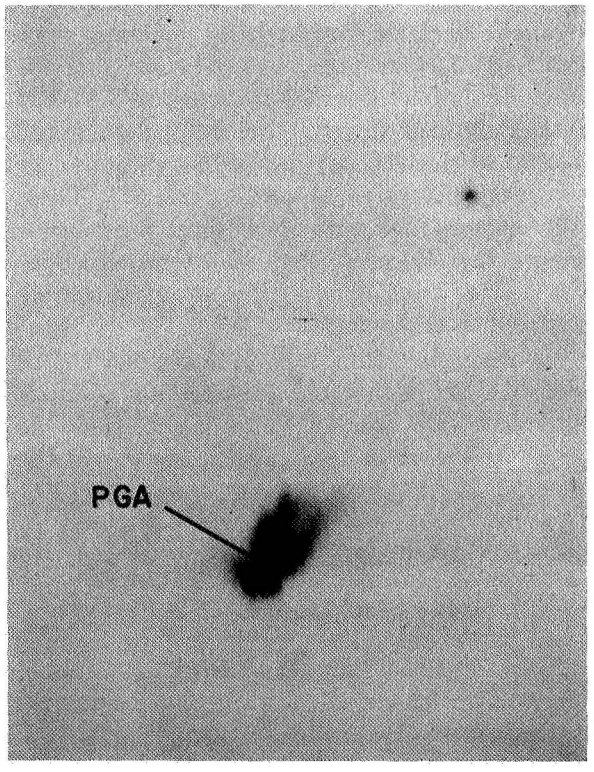

FIGURE 6.—Radioautogram prepared by exposing a two-dimensional paper chromatogram to Eastman Kodak No-Screen X-ray film. An aliquot was removed after a typical incubation to assay for Ru5–P kinase, chromatographed and the chromatogram then radioautographed.

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modes of growth might have been due to repression or the accumulation of inhibitor(s). If inhibitor(s) had accumulated in excess over the enzyme activity of interest, mixing of extract from cells having suppressed activity with extract from cells of higher activity would result in non-additivity of enzyme units. Data in table VII provide presumptive evidence that the decreased specific activities of both RuDP carboxylase and Ru5-P kinase were due to repression.

Results shown in table VIII reveal that Ru5–P kinase and ATP-, NADH-dependent CO_2 fixation are similarly inhibited by AMP, providing further evidence that reductive, ATP-dependent CO_2 fixation occurred via the Calvin cycle. AMP has essentially no effect on the RuDP carboxylase-catalyzed reaction. It is evident also that ADP inhibits CO_2 fixation and Ru5–P kinase.

In conclusion, the data suggest that a significant fraction of the CO_2 fixed during ribose oxidation by *H. facilis* is assimilated via the Calvin cycle. Suppression of CO_2 fixation into phosphoglycerate qualitatively parallels repression of the synthesis of RuDP carboxylase in ribose-grown cells. Data obtained with extracts from *H. facilis* grown in a variety of ways implicate levels of the carboxylase in the regula-

TABLE VII.—*RuDP Carboxylase and Ru5–P Kinase Activities*[a] *in Mixed Extracts*

Culture of origin and amount of protein (mg)	Activity (count/5 min)	
	Observed	Expected if additive
RuDP Carboxylase:		
Autotrophic (0.1)	7540	--------
Ribose (0.1)	1566	--------
Acetate (0.1)	17	--------
Autotrophic (0.05) + acetate (0.05)	3805	3779
Ribose (0.05) + acetate (0.05)	720	792
Ru5–P Kinase:		
Glucose (0.1)	1674	--------
Lactate (0.1)	957	--------
Glucose (0.05) + lactate (0.05)	1320	1316

[a] All the activities are corrected with those for controls which lacked substrate(s).

tion of CO_2 flux through the Calvin cycle during heterotrophic growth. Another catalyst that may regulate this flux is hydrogenase, the specific activity of which is suppressed by heterotrophic growth—as is well known from the reports of a number of laboratories. Hydrogenase may maintain saturating levels of reductant of CO_2 during autotrophic growth. It would be interesting to see whether hydrogenase and RuDP carboxylase are coordinately regulated.

Superimposed upon the regulation described may be control of CO_2 fixation through feedback inhibition by adenylate or ADP. Since CO_2 fixation requires ATP, the present observation is in accord with other adenylate effects (ref. 14). Thus, as the ATP/AMP+ADP ratio falls, cells would be afforded a mechanism to conserve ATP. Inhibition of CO_2 fixation by AMP or ADP using extracts of certain chemosynthetic and photosynthetic species has been reported (refs. 15 and 16). On the basis of the present work and another recent report (ref. 17), it is clear that one locus of the inhibition is the step catalyzed by Ru5–P kinase.

It is of major interest from a comparative biochemical viewpoint that in *H. facilis* and the two other chemosynthetic bacteria examined to date (refs. 18 and 19) NADH appears to be the reductant of CO_2. The same is true in photosynthetic bacteria (ref. 20). By contrast, work in a host of laboratories has indicated that the O_2-evolving photosynthetic species use NADPH as the reductant of CO_2. Horecker has suggested that use of NADH in reductive biosynthesis may be a very primitive property (ref. 21). These observations, then, provide support for the conjecture that O_2-producing photosynthetic species evolved from chemosynthetic and anaerobic photosynthetic bacteria.

In conclusion, it is well to remember that a mutation of hydrogen bacteria to obligate heterotrophism during space flight would be lethal to the passenger. This underscores the

TABLE VIII.—*The Effect of AMP and ADP on RuDP Carboxylase, Ru5–P Kinase and ATP-, NADH-Dependent CO_2 Fixation*

Conditions	RuDP carboxylase[a]	Ru5–P kinase[a]	ATP-, NADH-dependent CO_2 fixation		
			Cultured autotrophically	Cultured on lactate	Cultured on fructose
Complete	[b] (100)	[b] (100)	[b] (100)	[b] (100)	[b] (100)
− substrate(s)	1. 4	3. 9	1. 3	2. 4	3. 6
+ 0.01 *mM* AMP	----------	86	----------	----------	----------
+ 0.1 *mM* AMP	----------	55	92	----------	----------
+ 1 *mM* AMP	94	27	32	30	31
+ 0.1 *mM* ADP	----------	69	----------	----------	----------
+ 1 *mM* ADP	----------	25	----------	----------	46

[a] Extract from fructose-grown cells was used.
[b] Count/min incorporated by the complete systems from left to right were: 2706, 1343, 17486, 18840, 2065.

importance of a thorough understanding of autotrophism and heterotrophism.

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COMMENTS

Dr. JOHNSON. I am curious why you referred to the fixation which you achieved when nitrogen replaces hydrogen. Why do you refer to this as a heterotrophic fixation?

Dr. MCFADDEN. I think I referred to it as the so-called heterotrophic fixation. This is the term we used that proved to be clearly inadequate. When you are taking autotrophically grown cells, they are making storage materials; they can fix some CO_2 that is probably coupled with degradation of storage material. They also fix it by a mechanism that is identical, apparently, with that functioning under strictly autotrophic conditions.

The interpretation of experiments is difficult. I would only say that with *H. facilis* Brues' group has shown, under conditions of steady-state chemosynthesis, that PGA labels as if it is a major early product. In experiments as short as 5 seconds he was able to get, as I recall, up to 40 or 50 percent of the label in phosphoglycerate. The kinetics definitely indicated that it was decreasing with time to some steady-state level.

I do not think this question can be answered at the moment.

Enzyme Systems and Electron Transport

ROY REPASKE
National Institutes of Health
Bethesda, Maryland

Dr. McFadden has reported on some biochemical differences and similarities in carbohydrate metabolism of autotrophically and heterotrophically grown *Hydrogenomonads*. We are studying the electron-transport system of autotrophically grown cells and have made some preliminary observations on the electron-transport system of heterotrophically grown cells. These kinds of data on CO_2 fixation, carbohydrate metabolism, and terminal electron-transport systems will provide a basis for determining the effect of nutrients on the metabolism of *Hydrogenomonas eutropha*. The data may also give us information that permits some control of the organism's metabolism, thereby allowing some latitude in growth conditions but still making these organisms fulfill their role in a bioregenerative system.

In a sense these data will give us information about selected metabolic pathways at two extremes, that is, autotrophic and heterotrophic growth. We should not neglect the transition period, i.e., the time during which the cell will be committing itself to a change in substrate. I suggest that metabolic studies be supplemented with physiological studies to determine: (1) what concentration of organic metabolites induces heterotrophic growth, (2) what quantitative effect can be expected on gas utilization and cell growth during the changeover, and (3) how much time is required after depletion of the organic substrate before heterotrophic cells reestablish autotrophic metabolism. Since this is our first formal presentation of results at our annual conferences, my discussion will include a summary of earlier data.

Figure 1 shows some of the reactions we have studied in cell-free extracts of autotrophically grown *H. eutropha*. These reactions can be coupled to provide a model oxyhydrogen reaction. There are reasons for reserving judgment as to whether these reactions describe the physiological electron-transport system of the intact cell. Some of these reasons will be apparent during the discussion of particular reactions.

We had studied in some detail the hydrogenase coupled to pyridine nucleotide reduction. Flavin mononucleotide (FMN) was found to be specifically needed; neither flavin adenine dinucleotide (FAD) nor riboflavin were adequate substitutes for flavin mononucleotide (FMN); diphosphopyridine nucleotide (DPN), but not triphosphopyridine nucleotide (TPN), served as electron acceptor. Hydrogenase could also be assayed by methylene-blue reduction; this reaction was unaffected by the addition of FMN. In both assays the electron acceptors were reduced at similar rates. We had to determine whether the hydrogenase mediating methylene-blue reduction and the hydrogenase coupled to DPN reduction were different or the same hydrogenases (table I). If there were two hydrogenases, one would expect that when both electron acceptors were added simultaneously each acceptor would be reduced at the rate at which it was reduced when it was the only available acceptor. It is obvious that both DPN and methylene-blue reduction were inhibited when they were combined. Furthermore, under these conditions DPN could compete for electrons more effectively than methylene blue. These results suggested that the two acceptors shared a common electron donor, as shown in figure 2. The symbol X is undefined and may represent

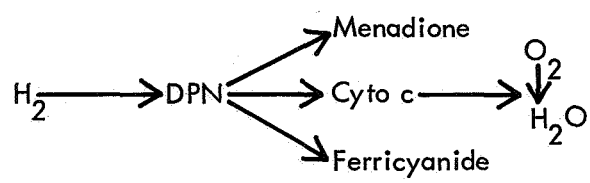

FIGURE 1.—Scheme 1.

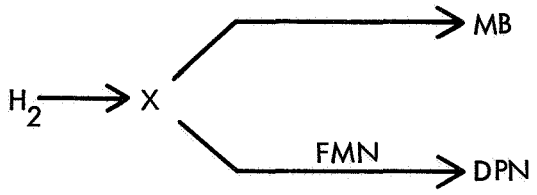

FIGURE 2.—Scheme 2.

TABLE I.—*Simultaneous Reduction of Methylene Blue and DPN*

Electron acceptor (pH 8.5)	mμ	ΔOD per minute	Inhibition (%)
DPN	340	0.034	
DPN+MB	340	0.027	21
MB	610	0.260	
MB+DPN	610	0.134	48

reduced hydrogenase or a reduced electron carrier.

It was observed that in the absence of FMN, ATP stimulated DPN reduction by molecular hydrogen when ATP and enzyme were preincubated together for 3 to 5 minutes (table II). Magnesium ions stimulated this reaction (table III). Note that ATP stimulation was not observed when FMN was present; additive rates are not obtained with saturating concentrations of FMN. A complete series of ribose tri-, di-, and mononucleotides were tested; only ATP and ADP give equivalent and significant stimulation. We cannot yet assess the role of ATP. ATP did not react with endogenous riboflavin and flavokinase to synthesize FMN, since added ATP and riboflavin had no additional effect over ATP alone. ATP did not participate in any reaction in which an exchange reaction with free phosphate was possible, because arsenate

TABLE II.—*Effect of Preincubation on ATP Stimulation*

Assay	ΔOD_{340} per minute
No addns	0.344
FMN	0.599
ATP (no preincubation)	0.304
ATP (with preincubation)	0.498

TABLE III.—*Stimulation on DPN Reduction by FMN and ATP*

Assay	ΔOD_{340} per minute
No addns	0.136
FMN	0.450
$MgCl_2$	0.124
ATP	0.210
ATP plus $MgCl_2$	0.308
FMN plus ATP	0.466
FMN plus ATP, $MgCl_2$	0.452

had no influence on the ATP stimulation. Uncoupling agents for oxidative phosphorylation had no effect.

The crude cell-free *H. eutropha* extract was placed on a Sephadex G–100 column. A fraction which followed elution of the cytochrome *c* band was slightly yellow in color and was required for ATP stimulation of DPN reduction by molecular hydrogen (table IV). As shown, the crude extract which was stimulated slightly by ATP was stimulated twofold, by this fraction and by ATP. The Sephadex fraction alone (not shown here) had no detectable hydrogenase activity by methylene-blue reduction or by the reduction of DPN with added FMN or added ATP. The Sephadex fraction and FMN clearly differed in their ability to couple hydrogenase with DPN reduction. The FMN concentration added to the crude extract was reduced to give approximately the same rate of DPN reduction as the added Sephadex fraction. Neither ATP nor the Sephadex fraction individually stimulated the rate with the suboptimal concentration of FMN. The expected stimulation by ATP plus the column fraction did occur in the presence of FMN, and it could be added to the FMN activity. Increased amounts of the column fraction plus ATP increased the rate of DPN reduction by the crude extract until the rate was equivalent to that obtained with saturating concentrations of FMN (table V). The rate was not exceeded by the addition of FMN. We therefore have what appears to be a second route for electron transport via an ATP-activated pathway to DPN.

We have preliminary evidence that a third pathway may exist for the routing of electrons

TABLE IV.—*ATP Stimulation by Crude Extract Supplemented with ATP and a Sephadex Fraction*

Assay	ΔOD_{340} per minute
Crude extract plus:	
No addns	0.100
ATP	0.124
Sephadex fraction	0.108
Sephadex fraction + ATP.	0.252
FMN (1.7×10^{-8} *M*)	0.138
FMN (1.7×10^{-8} *M*) + ATP.	0.180 (0.162 addition)
FMN (1.7×10^{-8} *M*) + Seph. fraction.	0.124
FMN (1.7×10^{-8} *M*) + Seph. fraction + ATP.	0.284 (0.290 addition)
FMN (6.7×10^{-7} *M*)	0.380

TABLE V.—*Maximum ATP Stimulation by a Sephadex Fraction*

Subject of test	ΔOD_{340} per minute	
	ATP assay	FMN assay
Extract:		
without addition	0.016	0.322
plus 0.1 ml Seph fraction	0.196	0.300
plus 0.2 ml Seph fraction	0.250	0.284
plus 0.4 ml Seph fraction	0.316	0.296
plus 0.6 ml Seph fraction	0.300	0.284
0.6 ml Seph fraction	0.016	0.020

from hydrogenase to DPN. This pathway does not require added FMN or ATP.

With the reduction of pyridine nucleotide, the cell has a reduced intermediate of low potential which is a common coenzyme required for many synthetic reactions. Tracing the electron transport system at this level is not simplified (fig. 1) because there are apparently three DPNH-oxidizing pathways.

With mammalian as well as with some bacterial systems, benzoquinones (Coenzyme Q or ubiquinones) or naphthoquinones (K vitamins) act as electron acceptors for reduced pyridine nucleotide. *H. eutropha* contains Coenzyme Q_8 but a reduced pyridine nucleotide (CoQ or Vit K reductase) has not been detected. We have found instead a very active benzoquinone and naphthoquinone reductase which is most active with unsubstituted quinones (table VI). The reductase is specific for reduced DPN and requires FMN for maximum activity.

TABLE VI.—*Quinone as Electron Acceptors for DPNH-Menadione Reductase*

Quinone	Conc. $\times 10^{-3}$ M	ΔOD_{340} per minute
		0.000
1,4 naphthoquinone	1.2	0.358
2-methyl-1,4-naphthoquinone (menadione).	1.2	0.040
2-methyl-3-chloro-1,4-naphthoquinone.	0.27	0.000
2-amino-1, 4 naphthoquinone	0.12	0.004
2-hydroxy-3-methyl naphthoquinone.	0.27	0.000
p-benzoquinone	1.2	0.490
methyl-p-benzoquinone	1.2	0.566

A DPNH cytochrome *c* reductase not requiring added quinones was also found. There is not enough *H. eutropha* cytochrome *c* yet available to determine whether natural cytochrome *c* behaves differently from the horse heart cytochrome *c* we have used in our assay. This reaction, too, requires FMN as a cofactor and it is stimulated additionally by FAD. Unlike menadione reductase, cytochrome *c* reductase is inhibited 30 to 50 percent by mono- and di-valent cations at concentrations of 10^{-2} M.

DPNH dehydrogenase (fig. 1) measured with ferricyanide as the electron acceptor may simply be the cytochrome *c* reductase which changes its characteristics because it is assayed with a different electron acceptor. This reaction, too, is stimulated by FMN; FAD has no effect. Unlike the cytochrome *c* reductase, however, it is not inhibited by mono- or di-valent cations. On column chromatography there is differential separation of DPNH reductase and DPNH cytochrome *c* reductase activities (table VII), which raises the question as to whether the enzyme catalyzing ferricyanide reduction is related to cytochrome *c* reductase.

In this series of reactions we have studied,

TABLE VII.—*Differential Elution of DPNH Dehydrogenase and Cytochrome* c *Reductase*

DEAE fraction	ratio $\frac{\text{DPNH dehydrogenase}}{\text{Cyto } c \text{ reductase}}$
1, 2, 3, 4	1. 1
5	8. 4
6	18. 7
7	23. 8
8	31. 4
9	26. 8
10	15. 8

only cytochrome oxidase is associated with particular elements of the cell. The cytochrome oxidase (fig. 3) is of the *o* type; the reduced spectrum has no absorption peaks in the 590- to 650-mμ region where cytochrome oxidases of the *a* type have typical absorption. A difference spectrum with reduced enzyme plus carbon monoxide (fig. 4) shows a carbon monoxide complex is formed with the reduced enzyme; this spectrum is typical of *o*-type cytochrome oxidases. Reduced cytochrome *c* oxidation by cytochrome oxidase is sensitive to carbon monoxide and cyanide; carbon-monoxide inhibition is partially relieved by light.

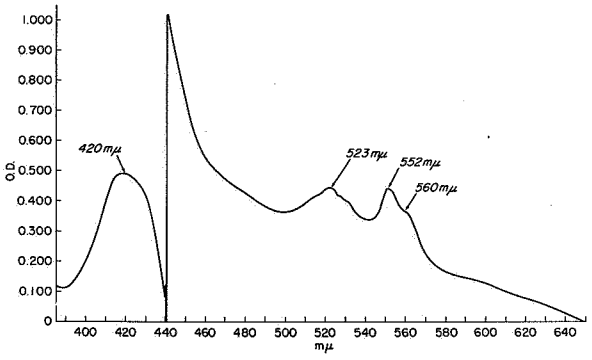

FIGURE 3.—Reduced spectrum, with cytochrome oxidase.

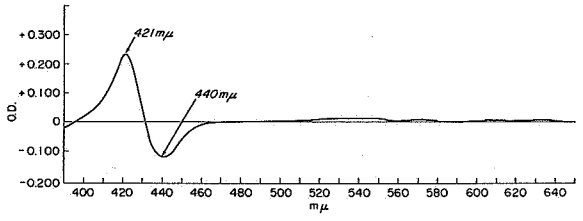

FIGURE 4.—Difference spectrum, with reduced enzyme minus reduced carbon monoxide.

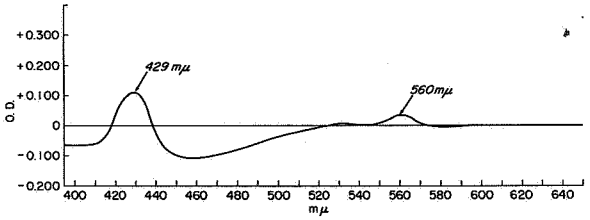

FIGURE 5.—Difference spectrum, with cytochrome b_1.

Another cytochrome, cytochrome b_1, is present in these organisms (fig. 5); it is also associated with particles of the cell. In mammalian electron-transport systems, cytochrome *b* is thought to participate between DPN and cytochrome *c*. Cytochrome *c* is rapidly reduced by *H. eutropha* extracts without the addition of cytochrome *b*. Virtually all of the DPNH cytochrome *c* reductase activity is recovered in the supernatant fluid after 2 hours of centrifugation at 140 000 g.

Heterotrophically grown cells have the same cytochrome complement as autotrophically grown cells. Cytochromes *c*, b_1, and *o* are detectable. Hydrogenase activity measured by methylene-blue or DPN reduction is less than one-fiftieth of that found in autotrophically grown cells. DPNH-menadione reductase from both types of cells has the same specific activity and the same characteristics of cytochrome *c* reductase; DPNH ferricyanide reductase and cytochrome oxidase have not been made. Preliminary findings suggest that heterotrophic growth affects the amount of hydrogenase present in the cells. However, this finding is not consistent with Bovells' observation (Ph.D. Thesis. University of California, 1957) that hydrogenase is a constituitive enzyme and, when measured manometrically with methylene blue, is virtually unchanged in heterotrophic cells. In addition, he finds that CO_2 has the same initial rate as both types of cells. The reasons for these discrepancies will be looked for.

COMMENTS

Dr. DECICCO. Were your heterotrophic cells grown with glucose only?

Dr. REPASKE. Also with Trypticase Soy broth.

Dr. JOHNSON. Was Bovell's organism *H. eutropha*, or did he use *H. facilis*?

Dr. REPASKE. No, he used *H. eutropha.* As a matter of fact, he is the one who isolated *H. eutropha* in the first place. Sometimes I am given credit for that, but I should not be. If you postulate that electrons travel as far as *B* by whatever mechanism, then there is auto-oxidation of *B.*

Dr. BONGERS. Why do you need *C*?

Dr. REPASKE. I do not know. The content of *C* is very high.

Dr. BONGERS. It is about equal to *B* in whole cells.

Dr. REPASKE. I noticed one thing that may or may not bear on our results—that is, your results and mine. When one reduces a preparation with hydrogen, cytochrome *c* is immediately reduced and cytochrome *b* comes up very slowly. Sometimes full reduction is not achieved for 5 or 10 minutes. This is not because oxygen has not been depleted in the cuvette. You can bubble hydrogen through the extracts, and if there is oxygen left, there would only be traces.

Dr. BONGERS. Which may be just enough to oxidize *B.*

Dr. REPASKE. Yes. But with additional hydrogen constantly there, if *B* is really reduced at all rapidly, and if it auto-oxidized rapidly, the reservoir of oxygen will be very low, and you would expect more rapid *B* reduction. But *B* reduction is terribly sluggish. I raise the question whether *B* is on the major pathway of electron flow.

Dr. MCFADDEN. From our studies with *H. facilis,* it may be of some interest to you that the CO_2 fixing system, which is dependent upon reducing power in ATP, appears to be in the soluble portion that we recover from high-speed centrifugation, after very brief sonication. This suggests, then, that perhaps the enzymes of the Calvin Cycle are available to use in reducing equivalents possibly generated by your soluble system.

Dr. REPASKE. I believe we have indirect evidence, too, that the cell knows when CO_2 is present, because it can grind away and oxidize hydrogen at a fantastic rate in the absence of CO_2. As soon as one provides CO_2, the cell knows that it had better start making ATP, and, it does. If ATP were made in the presence of oxygen and hydrogen, the amount of either phosphate or ATP available would soon be tied up as ATP, with no place to go, bringing the reaction to a halt. And, in fact, it does. It just keeps on cranking along in neutral. So, it does seem as if there is some kind of control between ATP formation in the presence of CO_2.

Dr. MCFADDEN. Do you attribute any physiologic role to ATP as an effector, that is, a possible effector of the hydrogenase activity that you are looking at as a generator of DPNH? It is a possibility, certainly.

Dr. REPASKE. It is premature to say anything about that. In the first place, the cell has an active hydrogenase that is stimulated by FMN; yet, there is not very much FMN available in the cell. In a way, this seems like a fluke. You have hydrogenase coupled with DPN, stimulated by FMN, with the cell having a very limited amount of free FMN available.

Dr. JOHNSON. I wonder if Dr. McFadden's finding that the DPNH stimulates an ATP-requiring enzyme could be tied in with this in some way.

Dr. REPASKE. I intuitively feel that there is going to be some connection between the two. And I thought the hydrogenase was simple! Suddenly it has become very complicated. I believe the picture is going to get more muddled before it gets clearer.

I did not show you data on the ATP system without added FMN. I get very good quinoquine perturbation, suggesting that riboflavin does not replace FMN. We have other evidence that *x* is perhaps not hydrogenase. If you store these extracts, this activity is lost. The methylene-blue activity is still retained. So it looks as if we have got something here in addition to FMN. A little longer storage and this drops out, too. But we still cannot get at the heart of *x.* We do not have a clue; or if we have it, we have overlooked it. We do not yet know what is beyond hydrogenase.

Dr. BONGERS. Have you any idea about the potential of *x*?

Dr. REPASKE. No, we cannot reduce methyl naphthoquinones. We can reduce benzoquinone; phenozene methly-sulphate is reduced. But our activity is highest with methylene blue. And it is most reproducible.

Metabolic Products of *Hydrogenomonas Eutropha**

ROBERT G. TISCHER AND LEWIS R. BROWN
Mississippi State University
State College, Mississippi

The first attempts made in our laboratories to determine the nature of the metabolic products of *Hydrogenomonas eutropha* were published by Brown, Cook, and Tischer in 1964 (ref. 1). Preliminary tests were made on the cell-free medium for carbohydrates, amino acids, and proteins; subsequently, many tests were made in efforts to characterize polybeta-hydroxybutyric acid (PBHBA) production. Absorption of C-14-labeled extracellular products, using two iron-exchange resins separately and together, indicated that the cation resin absorbed approximately 60 percent of the radiation; the anion absorption resin about 90 percent; and an equal mixture of resins, about 91.3 percent of the $^{14}CO_2$. Since approximately 60 percent of the radioactivity was absorbed by both the cation and anion exchange resins, with an additional 30 percent absorbed by the anion exchange resin, it was thought that approximately 60 percent of the metabolic products in the medium were amphoteric, while 30 percent were anionic. The remainder between this total and 100 percent is about 9 percent, and this quantity of metabolic products was considered uncharged. Originally ribose and the amino acids, glutamic acid, alanine, and tyrosine were identified in the extracellular material. Subsequent work indicated that glucose, arabinose, xylose, and ribose are also products of *H. eutropha* metabolism. Tests on amino acids indicated that three are produced by *H. eutropha* and that the same organism can oxide or assimilate all three amino acids without impairing the ability of the organism to oxide hydrogen (ref. 2).

The general objective of the work currently finished or being undertaken is to define the types and amounts of carbohydrates, proteins, fats, salts, etc. in the spent medium due to the growth of *H. eutropha* cells, expressed in terms of the medium's possible use as human food. On this project most of the effort has been spent on the identification and quantification of the carbohydrates, both used and produced by *H. eutropha*. It is hoped that very soon we may be able to branch out into other areas for a more comprehensive definition of the metabolic products.

A list of sugars (ref. 3)—including arabinose, fructose, galactose, glucose, lyxose, mannose, rhamnose, ribose, sedoheptulose, sorbose, sucrose, xylose, and a control—were tested to discern whether *H. eutropha* would use any of these sugars as a carbon and/or an energy source. The results were extremely simple, considering that the test organism used only one carbohydrate to any appreciable extent. This carbohydrate was fructose; it was used either heterotrophically or autotrophically and apparently did not inhibit the use of hydrogen. It was observed that autotrophically grown cells exhibited an *RQ* value of about 0.4 after 90 minutes of elapsed time, while fructose grown cells established an *RQ* of 0.9 or almost the theoretical 1.0 in the same time.

Comparisons between fructose-grown cells and autotrophically grown cells indicated that the fructose-grown cells could oxidize fructose without any lag phase whatsoever, whereas the autotrophically grown cells required a lag period of some 40 to 60 minutes before they could begin oxidizing fructose at anything like the rate exhibited by the fructose grown cells. Further studies indicated that *H. eutropha* metabolized fructose wholly or partly by the Entner-Doudoroff pathway.

When glucose is autoclaved under some conditions, it apparently breaks down into fructose which may then be employed by the *H. eutropha*

*The methods used in attaining the ends explained in this paper have all been published or exist in doctoral dissertations which are referenced at the end of the paper.

cells. This was disturbing news. Detailed tests of the effect of pH on the autoclaving of glucose solutions indicated that a pH values of from 3.0 to 6.0 there was very little change in color and very little oxygen consumption when these solutions were supplied to resting cell suspensions of *H. eutropha*; thus indicating that very little fructose was produced at these pH values. On the other hand, the color of the glucose solutions autoclaved at pH's 6.0 to 10.0 changed to yellow or brownish-yellow, and the oxygen consumption of resting cells with these solutions was markedly increased from 20–30 μl to 200–400 μl in 90 minutes, thus indicating that at higher pH's, fructose is apparently produced from glucose during autoclaving.

Up to the present time, ribulose 1,5 diphosphate + CO_2, if not the only method of introducing CO_2 into 3 phosphoglyceric acid, has been one of the most common. Having established the production of PGA by the organism, we have also established that PBHBA, when starved of nitrogen, is stored rather in the way that fat is stored in the human body. The PBHBA may, perhaps, be used up by the organism as fats are used by the human body under stress conditions such as very low temperatures or those conditions encountered during space travel. The remaining important alternative to the disposition of PGA in the present system appears to be glucose, possibly through one of the fructose intermediaries.

This pathway is not known, but it is known that ribose, xylose and arabinose, all of which are pentoses, are produced by some process that could go roughly in this direction. Incidentally, these pentoses are probably nowhere near as digestable by human beings as is the glucose that is produced but not used by the organism. Observation of our simple final diagram, then should indicate that we have available a very small amount of glucose, which should be readily available for human food. Steps evidently should be taken to maximize the production of glucose and similar carbohydrates that can readily be used by humans for food but not by the *H. eutropha*. It is clear also that the production of pentoses ribose, xylose, and arabinose, should probably be reduced in favor of more glucose production.

PBHBA and cells may be an excellent substitute for conventional foods for humans under the stress conditions of space, and the maximization of its production and the production of glucose, either by direct, indirect, or sequential methods, may submit to further experimentation. Therefore, in future research we must try to maximize the production of glucose and similar edible substances, investigate the pathways from carbon-dioxide fixation (possibly through PBHBA to glucose) and find one or more mutant strains of *H. eutropha* that could maximize the human food value of both the metabolic products and the organism itself. Expansion of this area of knowledge will necessarily include studies of acidic, amphoteric and neutral organic metabolic products and a consideration of the simple and complex salt effects that may arise in the re-use of a rejuvenated, spent medium.

The information exhibited in figure 1 is a composite of information from Cook, Kennedy, and Sultan (refs. 4, 5, and 6), plus some observations and speculations made in conjunction with a survey of the existing literature. From this diagram, it seems readily apparent that ribulose diphosphate plus carbon dioxide produces two units of 3 phosphoglyceric acid which may then go in the direction of carbohydrate or PBHBA.

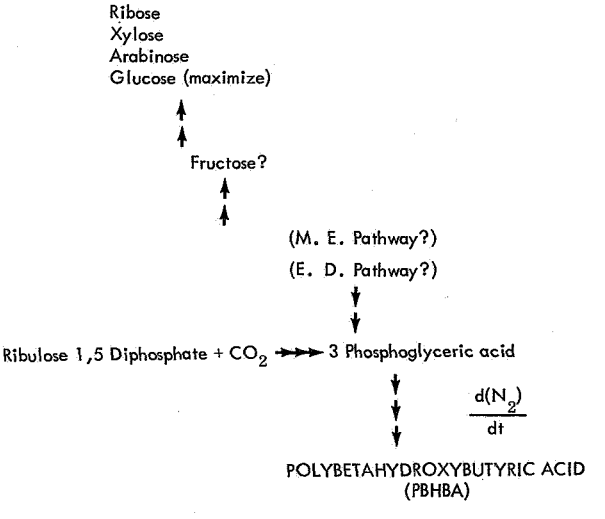

FIGURE 1.—Possible metabolic pathways from autotrophic conditions to human food.

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COMMENTS

Dr. REPASKE. I tried to grow *H. eutropha* on glucose. After my culture had been shaking for at least 2 weeks, I finally got some trace of growth, after which the growth was luxuriant. The organism grew very well on glucose and still looked like *H. Cutropha.* But it was brilliant yellow and it would not revert to autotrophy. It would repeat its subculture in glucose, but it lost all of its autotrophic ability. If this is a general kind of thing (perhaps in an environment in which you have free glucose in the medium as a waste product), you may just get a mutant of this kind and overgrowth and soon find that you have a heterotrophic population that will not revert to autotrophy. I raise this as a possibility.

Dr. DECICCO. We have done some work with glucose as well. As Dr. Repaske said, you can obtain mutants of *H. eutropha* which will grow in glucose. I was amazed. For once our results agreed with those of everyone else, specifically Dr. Repaske's. Bovell, I believe, also grew the organism on glucose. It takes between 7 and 14 days for your culture to come up if you innoculate at a very low level, about visible turbidity. The organism is still *H. eutropha*, as evidenced by phage studies. The organism grows very, very slowly on glucose, although you get very good cells yields. The best generation time (this is in shake flasks at low densities, which would be your maximum growth rate) is about 11½ hours. These will keep going, and you will reach optimum densities in shake flasks of up to 14, which would be roughly about 5 milligrams dry weight per milliliter or about 5 gram per liter.

One unusual characteristic of the population we studied is that half of the cells, after growth on glucose at different intervals of time, are glucose-utilizing organisms. The growth rate is very slow. It appears that the wild-type cells (normally non-glucose-utilizing) can live off the glucose-utilizing organisms. The pH drops considerably. Thus, the glucose-utilizers are probably pumping out some organic acids which the wild-type organisms can then utilize for growth.

When I checked these cells, only 20 percent of the population were glucose-utilizers. The other 80 percent presumably were growing off the glucose mutants. We purified the glucose mutants and checked these for a few characteristics. Pure glucose utilizers (they had hydrogenase more active than any other we found) grow autotrophically.

Dr. REPASKE. They do grow autotrophically? How did you grow them in glucose, under gases or in air?

Dr. DECICCO. In air.

Dr. REPASKE. We will have to exchange cultures.

Dr. DECICCO. The other thing is, we usually use from 0.2- to 0.4-percent organic substrates when we culture the organisms heterotrophically. They grow much better at 1 percent. It appears to be impermeability, as you would expect, to an impermeability mutation—a mutatiton of the transport system. The wild-type culture is cryptic with respect to glucose. If you grow them in 1-percent glucose, the cells in culture would come up much faster. In some cases you do not get growth at all at 1 or 2 percent.

The mutation rate is fairly high. I would expect the mutation rate to be about 10^{-6} or 10^{-7}; in other words, about one organism is about 10^{6} or 10^{7}; will be the mutant that will occur. The problem is not that the glucose-utilizing mutant is hard to come by. More important is the fact that it grows so slowly on glucose that even after it comes up, you get one mutation occurring that is not going to reach turbidity for another 8 to 10 days. By this time, usually you have wiped out your experiment.

Dr. TISCHER. Then by replenishing your culture from other circumstances you should be able to hold that in tow, should you not? If I understand correctly, Dr. Repaske got his yellow organism—which I hope you have a culture of—after 2 or 3 weeks on a glucose medium; is that right?

Dr. REPASKE. The autotrophic medium supplemented with glucose was grown in air.

Dr. TISCHER. Then it would seem to me that what we need to know for our situation is what happens in the situation we are planning to use—that is, growing it in hydrogen rather than in air. In other words, would the same situation prevail there as it does in air? If not, then it is not a problem. If it does, quite obviously maximizing glucose output would have some extra difficulties built in.

Dr. DECICCO. By glucose-utilizing mutants I am re-

ferring to organisms that show growth on glucose. I feel that the wild-type culture can take glucose in very slowly. Here is where your concentration effect is probably evident. They probably can take in enough glucose to maintain themselves. Our wild-type culture grows very, very slowly. Before the mutants have a chance to come up, there is a generation time of probably 24 hours or more.

Since it is a transport system, this is not unreasonable. Glucose may penetrate very, very slowly and enable the cells to obtain enough energy for maintenance and slight growth.

Dr. TISCHER. This probably would be a survival mechanism.

Dr. DECICCO. Yes, possibly.

Dr. TISCHER. If that is all it is, and it only goes on at that rate, it seems to me we ought to be able to circumvent that whole situation without too much trouble.

Dr. DECICCO. I do not know how you would get around the glucose rapid-utilizing mutants, which are another problem.

Dr. TISCHER. We do not have any of those to date, do we?

Dr. DECICCO. Yes, we have. These are the ones that come up in 7 to 14 days. You get this burst, which is probably what you observed. Eventually you get rapid growth. Very low turbidity for a long time, and then they start up fairly rapidly. Upon subculturing, these will keep on growing at about this rate; a doubling time of 10 or 11 hours.

Dr. TISCHER. Is your work in air also?

Dr. DECICCO. This is in air. We have done some work with some other compounds under what we call simultaneous conditions. I do not know if we have done anything with glucose under those conditions generally.

Dr. TISCHER. I do not see how this would affect the circumstances I was describing, because this one would be in hydrogen.

Dr. DECICCO. The question is are they going to use glucose when they are growing in hydrogen? We have information using other organic compounds including fructose. This may have a bearing on it.

Dr. TISCHER. It obviously needs some solution.

Dr. MCFADDEN. How are you sure that the cells are excreting glucose and that this is not just a reflection of lysis?

Dr. TISCHER. I do not suppose that at this point we are entirely sure of this. It is a rather difficult thing to get at, is it not? Lysis is occurring even during the most rapid rate of growth. This cyclic sort of thing is like the insides of photosynthesis and respiration. It is pretty hard to separate them and find out something about one and not about the other in a compounded fashion. I am afraid I cannot answer your question.

Genetic Stability of *Hydrogenomonas Eutropha*

Benedict T. DeCicco
The Catholic University of America
Washington, D.C.

Population changes during long-term cultivation of "pure" bacterial cultures are well known. These were first observed and studied during the early 1900's and were explained by various concepts, including cultural adaptation, orderly life cycles, or a combination of such ideas. Although the mutation and selection theory was accepted by most biologists shortly after it was proposed around 1900, the majority of microbiologists rejected the application of these ideas to microorganisms until the later 1940's. The classical experiments of Luria and Delbruck (1943), Newcombe (1949), and the Lederbergs (1952) laid the foundation for the acceptance of mutation as a major cause of population changes during bacterial growth. These population shifts were observed during subculturing in both liquid and solid media and were later studied using the chemostat and the turbidostat, where such shifts are readily observable.

The mechanisms behind population changes in microbial cultures have been investigated in the past few decades, and many causes for such changes have been reported. These mechanisms involve factors allowing for the selection of certain cell types in the population rather than others. When large cell populations are studied, such as the 1 to 10 trillion hydrogen bacteria usually obtained per liter of medium, a genetically pure bacterial culture is not possible. Because of spontaneous mutations, the number of different cell types present under these conditions is very large. Since the occurrence of these mutations cannot be controlled, conditions must be maintained that will prevent any unfavorable cell types from reaching sizeable proportions.

Some factors allow the complete selection of mutant cells in a population. This selection commonly occurs during phage infections, since only phage-resistant cells can survive and the progeny of these will eventually repopulate the culture. The same situation could arise by phage infection of a *Hydrogenomonas* life-support system. The important factors here are phage infectivity under the specific cultural conditions; the rate of repopulation by uninfected or resistant cells; and the physiological behavior of the new population, especially with respect to growth rate, gas consumption, and conversion efficiencies.

A quantitative selection of mutant cells often occurs as a result of medium composition. This may, in turn, result from the production of self-inhibiting extracellular products by wild-type cultures, which allow growth of cell types resistant to the products. The isolation by Dr. Tischer's group of extracellular products during autotrophic growth of *H. eutropha* suggests that such products may prove inhibitory when sufficiently high cell densities are studied.

Perhaps more important are selective forces imposed by the initial composition of the medium, especially when large amounts of undefined organic compounds are present. The use of a complex material like urine in a system designed specifically for autotrophic growth requires considerable attention. The effect of organic substances upon the autotrophic metabolism of wild-type cells must be considered. The high organic content of a urine-supplemented medium could also enable variants of *Hydrogenomonas* with impaired autotrophic capacities to thrive, and it is quite possible that variants with increased heterotrophic capabilities might be somewhat deficient, or less efficient, in their autotrophic systems. The overall result could be decreased H_2 and CO_2 consumption and increased O_2 demands.

STABILITY OF THE AUTOTROPHIC SYSTEM

In addition to the variations encountered among genetically distinct cell types, one must

also consider the range of variation possible within a single cell type and the variation type that can produce more rapid fluctuations in cultures. We therefore wanted to know the range of activity of a presumably normal *H. eutropha* culture during cultivation under a given set of conditions. As one measure of activity, hydrogen oxidation was observed as a function of culture age under strictly autotrophic conditions. For these experiments, flasks containing Bongers' salts medium (ref. 1) with urea were inoculated to just visible turbidity and observed for 6 days. The gas atmosphere was 70-percent H_2, 20-percent O_2, and 10-percent CO_2. Hydrogenase activity was measured manometrically at various growth stages throughout this period (table I). Activity is expressed per OD 10, which refers to 1 ml of OD 10 culture per Warburg vessel; this corresponds to 3.5-mg cells (dry weight) per vessel. It is apparent that hydrogenase activity is quite constant between the late-lag and late-log growth phases. Activity then gradually declines until, at the sixth day, the activity is about one-fourth of its mid-log phase value. This lowered activity does not appear to be caused by a high proportion of dead cells since, as seen below, activity is rapidly restored upon subculturing.

TABLE I.—*Hydrogenase Activity of Autotrophically Grown* H. Eutropha

Time (days)	Growth phase	μl H_2 consumed/min/OD 10
1.0	Late lag	17.0
2.0	Mid log	21.4
2.5		18.6
3.0	Late log	17.2
4.0	Early stationary	11.5
6.0	Stationary	5.6

Table II shows the effect of inoculating a stationary phase culture of autotrophically grown *H. eutropha* into media containing various organic substrates. The cultures were incubated under air for 7 hours to allow the cells to undergo one and a half to two divisions and were then assayed for hydrogenase activity. The data demonstrate that the hydrogenase activity of *H. eutropha* is not repressed by the tested organic compounds. In fact, although a stationary-phase autotrophic culture with low hydrogenase activity was the inoculum, the activity under heterotrophic conditions rapidly increased to about the maximum autotrophic level. Thus *H. eutropha* clearly contains a constitutive type of hydrogenase.

TABLE II.—*Hydrogenase Activity of Autotrophically Grown Cells After Culturing in Organic Media for 7 Hours*

Substrat	Hydrogenase activity (μl gas/min/OD10)
Autotrophic inoculum	5.5
Fructose	22.5
Phenylalanine	20.1
Trypticase soy broth	17.4
Glutamate	25.4

We had previously reported an inhibition of hydrogenase activity of *H. facilis* by certain organic compounds (ref. 2). *H. facilis*, however, contains an inducible hydrogenase and might be expected to show such a repression. With *H. eutropha*, the effects on the autotrophic or heterotrophic systems when both types of substrate are available were studied by observing the growth of the organism under these "simultaneous" conditions and correlating this with measurement of pH, hydrogenase activity, and remaining organic substrate. For the simultaneous experiments, glutamate was chosen as the organic substrate since this allowed the most rapid heterotrophic growth of any single compound tested. With 0.4-percent glutamate in Bongers' salts, the doubling time for *H. eutropha* at 30°C is about 2 hours. Cultures were grown in 0.4-percent glutamate under air or under H_2, O_2, and CO_2 and compared to growth under strictly autotrophic conditions. Hydrogenase activity, as expected, was similar for mid-log phase cultures under all three conditions, varying between 19 and 22 μl gas consumed/min/OD 10.

The most interesting effect of the comparative growth study was a consistently observed growth inhibition under simultaneous condi-

tions. This inhibition occurred shortly after inoculation and was soon reversed. Growth then proceeded in both flasks at the same rapid rate, giving parallel curves. Thus it appeared that the presence of the autotrophic gas mixture inhibited the utilization of glutamate.

Gottschalk (ref. 3) has reported an inhibition of the utilization of some organic compounds by a hydrogenomonad similar to *H. eutropha*. In his system a complete stoppage of growth was observed in media containing fructose or glutamate when the gas phase was H_2 and O_2. He stated that the presence of H_2 repressed the induction of the heterotrophic system. If CO_2 was added to the gas mixture, the repression was reversed. We checked our system to see if H_2 and O_2 would either temporarily or permanently inhibit growth (table III).

It can be seen that at 3.75 hours the ratio of growth in the glutamate plus H_2, O_2, and CO_2 medium, compared to the glutamate medium under air, is .07/.11 or about two-thirds. This ratio remains constant for all subsequent readings throughout the exponential growth phase. The inhibition is extremely reproducible, and growth under H_2, O_2, and CO_2 is always about 66 percent of growth in glutamate plus air from 4 hours post-inoculation through the log phase. This means that, within the first 4 hours of incubation, the culture under the autotrophic atmosphere is inhibited for a period equal to two-thirds of a generation cycle or a period of 1⅓ hours. Table III demonstrates that little growth and no inhibition occurs within the first 1½ hours of incubation and that, after 3¾ hours, the multiplication factors for the three glutamate flasks are very similar. Thus a 1⅓-hour inhibition period occurs in the glutamate plus H_2, O_2, and CO_2 flask between 1½ hours and 3¾ hours post-inoculation. This is seen during the time interval in the multiplication factors that show a 2.7-fold increase under air or H_2 plus O_2 but only a 1.7-fold increase under the complete autotrophic atmosphere.

Thus H_2 is not an inhibitor in this system, but either CO_2 or CO_2 plus H_2 is. Table IV

TABLE III.—*Growth of* H. Eutropha *Under Autotrophic, Heterotrophic, and Simultaneous Conditions*

Incubation time (hr)	0.4% glutamate + air OD_{540} × factor		0.4% glutamate + H_2, O_2, N_2 OD_{540} × factor		0.4% glutamate + H_2, O_2, CO_2 OD_{540} × factor		Salts + H_2, O_2, CO_2 OD_{540}
0	0.03		0.03		0.03		0.04
1.5	0.04		0.04		0.04		0.04
3.75	0.11	2.7	0.11	2.7	0.07	1.7	0.05
5.0	0.15	1.4	0.15	1.4	0.10	1.4	0.05
7.5	0.35	2.3	0.31	2.1	0.22	2.2	----------
11.5	2.0		1.6		1.2		----------
28.5	3.2		3.2		3.2		1.2

TABLE IV.—*Inhibition of Heterotrophic Growth by Carbon Dioxide*

Incubation time (hr)	0.4%-glutamate +air OD_{540}	0.4%-glutamate +H_2, O_2, CO_2 OD_{540}	0.4%-glutamate +H_2, O_2, N_2 OD_{540}	0.4%-glutamate +N_2, O_2, CO_2 OD_{540}
0	.015	.015	.012	.015
2.5	.050	.041	.043	.039
4.5	.060	.055	.062	.050
7.0	.143	.109	.140	.090
8.5	.180	.125	.178	.135
10.5	.29	.18	.28	.18
12.5	.47	.26	.44	.25
14.5	.87	.50	.80	.48

demonstrates that CO_2 alone is responsible for the observed inhibition. Growth under H_2, O_2, and N_2 is similar to growth under air, while N_2, O_2, and CO_2 (or air plus CO_2) show an inhibition pattern similar to that observed with the complete autotrophic atmosphere.

In addition to the temporary inhibition of growth during simultaneous autotrophic and heterotrophic conditions, several other aspects of growth under these conditions were revealed by extending the previously mentioned experiments over a longer period. (fig. 1). The temporary inhibition when glutamate, H_2, O_2, and CO_2 are available can be seen as an increased lag. The growth curves are then parallel with all three atmospheres. The glutamate plus air and glutamate plus H_2, O_2, and N_2 flasks yield maxima at optical densities between 3.5 and 4.0, and glutamate measurements show that glutamate is exhausted when each culture reaches its peak. Under air the OD then decreases fairly rapidly, presumably because of cell autolysis. When a H_2, O_2, and N_2 mixture is present, the OD remains quite constant near the peak level for a considerable time, suggesting that energy derived from H_2-oxidation can be used under starvation conditions to maintain cellular integrity.

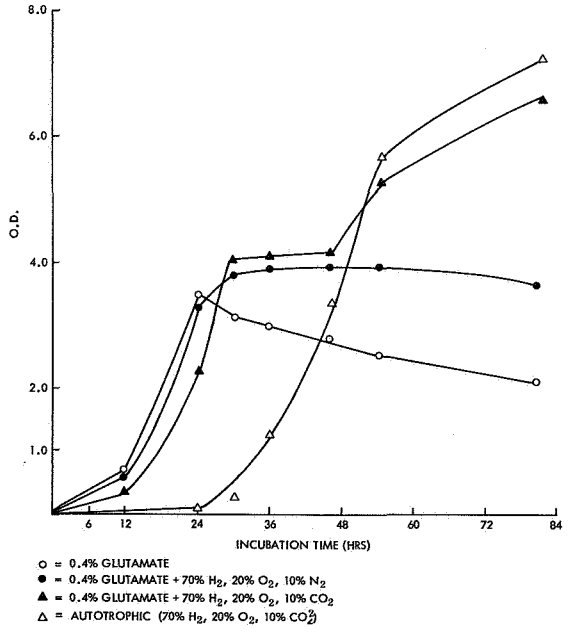

FIGURE 1.—Growth of Hydrogenomonas eutropha under autotrophic, heterotrophic, and simultaneous conditions.

The culture incubated under simultaneous autotrophic and heterotrophic conditions reaches a temporary maximum because of glutamate exhaustion at a cellular density slightly greater than the other glutamate cultures. Therefore, growth using both autotrophic and heterotrophic mechanisms simultaneously does not occur to any great extent under these conditions. The temporary peak forms a plateau that extends for about 10 hours, after which growth resumes autotrophically. This diphasic growth pattern is characteristic of the "diauxie" effect—the sequential utilization of one substrate and then another. The first substrate usually supports faster growth, while the second is often catabolized adaptively; thus, the plateau is usually the period required for induction of a catabolic system. In this case the substrate used first (glutamate) does support faster growth, and the plateau period may be the time required for the induction of some component of the autotrophic system.

H. EUTROPHA *BACTERIOPHAGES*

Very little is known about phage infection of autotrophic bacteria. The first phage to be studied, which is active against an autotrophic bacterium, was a *Hydrogenomonas facilis* phage isolated in 1962. The *H. facilis* phage showed fairly good activity in organic media but little or no activity, as evidenced by cell lysis, in autotrophic media. The high-phage titers readily obtainable with many other phages could not be attained with *H. facilis* phages.

The opportunity to study phage active against other autotrophs led us to search for *H. eutropha* phages. These would be useful for genetic studies as well as for observing infections with a faster growing autotroph than *H. facilis*.

It was first established that *H. facilis* phages will not attack *H. eutropha*. We therefore assayed various soil, water, and sewage sources in the vicinity of Washington, D.C., and obtained three phage types active against *H. eutropha*. These phages did not produce the large, clear plaques characteristic of many of the enteric phages, and it was suspected that,

since our strain of *H. eutropha* was originally isolated from California, the phages we had obtained may be primarily parasites of some eastern relative of *H. eutropha*. We subsequently obtained a soil sample from TRW, Inc. Systems and isolated a fourth phage type (table V).

TABLE V.—Hydrogenomonas Eutropha *Bacteriophage Types and Their Origins*

Type	Origin
HeMa	Soil—Silver Spring, Md.
HeMb	Creek—Adelphi, Md.
HeW	Sewage Treatment Plant—Washington, D.C.
HeC	Soil—Redondo Beach, Calif.

The *HeC*-phage type appears to be more virulent than the other three, and fairly high titers are easily obtainable by infecting heterotrophic cultures. The three local varieties appear to be quite unstable. Only a few plaques obtained at any one time are of medium size and clear, while most are tiny and opaque. If the larger clear plaques are isolated and used for subsequent infections, there is a gradual reversion to the predominantly avirulent form. This is especially true for the two Maryland phages, types *HeMa* and *HeMb*; and it is, therefore, difficult to obtain high titers with these varieties. Type *HeW* shows higher virulence than the Maryland types and will also yield higher titers.

Cultures that are resistant to each of the four phage types were obtained by infecting about 10^8 cells of *H. eutropha* with a large number of each phage type on agar plates and isolating phage-resistant clones that eventually develop. These phage-resistant cultures were then checked for sensitivity to each of the four phage types (table VI). Each culture should, of course, be resistant to the phage type from which it was selected. The pattern of sensitivity or resistance to the other three types should indicate the relationship between phages.

As might be expected from other characteristics, bacteria resistant to either *HeMa* or *HeMb* exhibit cross-resistance to the other type. These two phages are probably closely related or identical. Surprisingly, type *HeW* shows a closer relationship to type *HeC* than to the Maryland strains.

TABLE VI.—*Cross-Resistance Pattern Among* H. Eutropha *Bacteriophages*

Culture	Phage type tested			
	HeMa	*HeMb*	*HeC*	*HeW*
H. eutropha resistant to *HeMa*	−	−	+	+
H. eutropha resistant to *HeMb*	−	−	+	+
H. eutropha resistant to *HeC*	+	+	−	−
H. eutropha resistant to *HeW*	+	+	−	−

+ = lysis.
− = no lysis.

Bacteria resistant to each of the four phage types rapidly revert to phage sensitivity upon subculturing. This, coupled with the lack of complete cross-resistance among the four types, renders as improbable the usefulness of employing a phage-resistant culture as inoculum for a life support system.

The effect of phage infection during autotrophic and heterotrophic growth has been examined. Under heterotrophic conditions the effects will vary from no noticeable change to complete clearing of the medium. This depends mainly upon the medium employed and the ratio of phage to bacteria. Under autotrophic conditions low numbers of *HeMa* or *HeMb* phages show no effect, but higher doses of types *HeW* or *HeC* will produce inhibition or lysis of an autotrophic culture as shown in table VII. Here flask 1 is an uninfected control and has increased to OD 0.66 during the 22-hour incubation

TABLE VII.—*Effect of Phage Infection During Autotrophic Growth of* H. Eutropha

Phage type	OD_{540}	
	0 hr	22 hr
	0. 10	0. 66
HeC	0. 10	0. 17
HeW	0. 10	0. 07

period, while flasks 2 and 3 show a definite impairment of growth during the same period. Further studies are needed under varying conditions and especially in urine-supplemented media, where a more pronounced effect is anticipated than under purely autotrophic conditions.

NUTRITIONAL MUTANTS OF H. EUTROPHA

The isolation and identification of nutritional mutants have been invaluable in studying various aspects of microbial physiology and metabolism. Such mutants can also be valuable in comparative studies of the autotrophic and heterotrophic mechanisms as well as for genetic experiments. We therefore developed a technique for obtaining auxotrophic mutants of *H. eutropha*, or mutants that have specific growth factor requirements. Using a modification of the penicillin technique 49, suspected auxotrophs were isolated and examined for their particular requirements. We were especially seeking amino-acid auxotrophs and found that seven of the strains would grow on a medium supplemented with casamino acids. The individual requirements were then determined (table VIII).

TABLE VIII.—*Auxotrophic Mutants of* H. Eutropha

Strain	Growth factor required
A–1	methionine.
A–12	histidine.
A–14	tryptophan.
A–16	methionine.
A–23	cystine.
A–24	phenylalanine plus tryptophan.
A–25	histidine.

Strain A–24 requires both phenylalanine and tryptophan and probably contains a single enzymatic block somewhere along the shikimic-acid pathway. This mutant is similar in its requirements to one we had obtained previously from *H. facilis*. Both organisms apparently can convert phenylalanine to tyrosine since tyrosine is not required. The *H. facilis* mutant requires para-amino benzoic acid in addition to the two amino acids, but we have not yet determined a similar requirement for strain A–24 of *H. eutropha*.

A CLOSED ENVIRONMENT CHEMOSTAT FOR CULTURAL STABILITY STUDIES

It is well established that a steady-state culture cannot be duplicated by repeated subculturing in broth or on agar. The population dynamics that may be encountered during growth under steady-state conditions are almost certain to escalate or, in some cases, be repressed in the uncontrolled environments afforded by a flask, tube, or plate culture. We have developed a chemostat that will function under a closed recirculating atmosphere and will allow rapid adjustments to be made in total gas pressure, partial gas pressures of the component gases, and medium flow rate. The system is simple in design and operation and requires a minimum of time and effort in preparation and maintenance (fig. 2).

The gas reservoir contains a H_2, O_2, and CO_2 mixture in the desired proportions. The mixture is circulated to and from the culture vessel through a small pressure-vacuum pump, with the gas reservoir acting as a circulating bypass for excess pump pressure. The two clamps leading to and from the gas reservoir enable both the amount of aeration and the gas pressure in the culture vessel to be regulated. A manometer

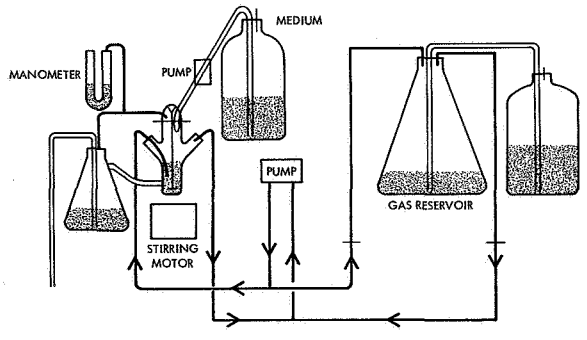

FIGURE 2.—Chemostat for cultivation of hydrogen bacteria.

is built into the system to allow the pressure in the culture vessel to be continuously monitored. The culture is aerated via a submerged sparger, and a magnetic stirring bar assists in further agitating the suspension. Media is fed from the media reservoir into the culture vessel by a peristaltic pump, and the flow rate is regulated by a cam timer.

At present the culture vessel being used has a 50-ml capacity, but a 250-ml vessel can be substituted if the need arises. The system has undergone a preliminary 7-day run with *H. eutropha* and, after minor adjustments, is functioning properly. We intend to observe both normal and mutant cultures of *H. eutropha* for population changes during prolonged cultivation before determining the effects of phage infection in this system.

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COMMENTS

Dr. JENKINS. Last year you were rather optimistic about the phage problem when you were talking about overgrowth of *H. eutropha* over the phages. What is your present assessment?

Dr. DECICCO. It has not changed. The mutation rate to phage resistance is quite high. We have not determined it exactly, but it would be at least 10^{-6}. Once you have reached the population of 10^6, you are going to have phage-resistant mutants present against any of these four phage types.

As you may know, once your culture increases above the level at which mutants first appear, the proportion of any particular mutant will increase. Thus, if you have a mutation rate to phage resistance of 10^{-6}, this does not mean that when you reach 10^9 you are going to have a thousand mutants present. You would probably have ten thousand or closer to a hundred thousand, because the ratio of mutants to wild-type cells increases linearly as your population increases.

It does not appear to be much of a problem even if all sensitive cultures were destroyed. Under most conditions this does not happen. Under most conditions it appears that all of the sensitive bacteria are not destroyed by phages. Again, I do not know whether you are getting lysogeny. We have attempted UV-irradiation of resistant cultures to see whether they were, in fact, lysogenic. We could not detect phage production. This does not, of course, rule out lysogeny; there is still, I would say, a very good possibility. It just means that it may not be induced by UV, or we may not be using the right system.

Normally in the check for lysogeny you need two strains of the same bacteria, one that becomes lysogenic with a particular phage and another that is sensitive to that same phage. Of course, we do not have two strains of the same organism. We have only one strain of *H. cutropha*. Perhaps, if we could get some of Dr. Schlegel's strains from Germany, or some other, we may be able to have an indicator strain for phage sensitivity.

In any event, repopulation occurs quite rapidly. I do not know what the time intervals would be. It would depend on your medium and how fast these organisms would grow. I can say that the phage-resistant mutants, as far as we know now, are essentially similar in most respects to those we have observed in the original culture. So, therefore, if you do have phage infection, you should get repopulation with predominantly phage-resistant culture. Eventually, this would probably revert to the normal wild-type, phage-sensitive culture. In this case your phages, as they normally do under our conditions, may eventually disappear. We do not know what happens to them.

Dr. JENKINS. Have any of the other investigators had problems with phage?

Dr. DECICCO. Under autotrophic conditions, I would not expect this to show up—for the simple reason that I am sure that, in an autotrophic culture with one phage or 10 phages, you are not going to see anything unless you get a phage that is certainly a lot more virulent than the ones we have studied. Because, as I said, we only notice an effect under autotrophic conditions when we are dealing with many phages.

On an agar plate with organic substrate, you can get a nice plaque developing from one phage particle. So here, presumably, one phage would do damage. But, under autotrophic conditions you do not get this type of a pattern. The phage deteriorates very rapidly.

Dr. REPASKE. When you say you have many phages, what multiplicity are you talking about?

Dr. DECICCO. I do not know exactly. It varies.

Dr. REPASKE. Would the autotrophic culture be affected?

Dr. DECICCO. I would say on the order of 1-to-1.

Dr. REPASKE. In table VII where you showed the phage?

Dr. DECICCO. Yes. These flasks contain 10 ml of medium. We used 0.1 ml of phage inoculum that presumably contained 10^7 or 10^8 phage particles. The culture was inoculated to just visible turbidity, which, again, would be about 10^8 bacteria per 10 ml. I would

say we were fairly close to a 1-to-1 multiplicity, give or take 1 log.

Dr. REPASKE. Did you only detect lysis when the culture was growing? Or did you detect lysis of a nongrowing culture? In the experiments here your culture was 22 hours old.

Dr. DECICCO. Yes.

Dr. REPASKE. From the initial time until you made your measurements?

Dr. DECICCO. Yes.

Dr. REPASKE. Now, if you had just taken a 10-hour-old culture, for instance, and added the phage, would you have obtained clearing?

Dr. DECICCO. You mean without allowing for further growth? Arresting cell suspension?

I would doubt it very seriously. This normally does not happen with phage infection. You have to have growing cells.

Dr. REPASKE. One thing that bothered me was the long lag period for your autotrophic growth.

Dr. DECICCO. Again, we are trying to study rather subtle effects. In order to do this we use low inoculum. If we use high inoculum, we do not get this lag. We get a lag of a couple of hours. But, when we inoculate a culture under autotrophic conditions to just visible turbidity, we observe a lag of about 16 to 18 hours before we start getting an increase. Has anyone else been working under this type of condition and observed a similar lag, with low inoculum, to just visible turbidity after inoculation, roughly, 10^7 cells per ml?

Dr. BONGERS. I think you would see a very short lag in a couple of hours at most.

Dr. REPASKE. When I start with an initial OD of about 0.1, and observe a 3-hour lag period, there is usually something wrong.

Dr. DECICCO. Is this with an autotrophically or a heterotrophically grown inoculum?

Dr. REPASKE. Autotrophic.

Dr. DECICCO. That's the difference.

Dr. COX. What do these phages look like? Do you know?

Dr. DECICCO. No. I haven't taken any pictures.

Dr. COX. Have you looked for any other indicator strains?

Dr. DECICCO. We have checked some other strains, including some *Pseudomonads*, and these are not sensitive.

Normally when you are going to do something like this, you suspect cultures that are related to your own. Very rarely do you get cross-infectivity of a single phage with two organisms showing very few similarities. The only cultures I know that appear to be related to *H. cutropha* are the cultures that Schlegel has been working with in Germany. Certainly *H. facilis* is not affected; but, on the other hand, *H. facilis* is not very similar to *H. cutropha*. They are very different.

Dr. BONGERS. I must have misunderstood. When you compared the glutamate grown cells heterotrophically to the autotrophically grown cells, did you start out with an inoculum that was originally grown from glutamate?

Dr. DECICCO. The table that I showed you was with a heterotrophically grown inoculum. We did the same thing with an autotrophically grown inoculum. You get exactly the same inhibition. The only difference is your strictly autotrophic culture comes up faster.

No one ever did work on simultaneous growth until a paper, by Goodman and Rittenberg about 3 or 4 years ago, at the American Society of Microbiology meeting. He was studying simultaneous growth in an organic substrate with hydrogen. He was working with lactate. He found that you did get simultaneous growth as measured by yield, greater yield under both conditions, as compared to lactate alone.

I think this is probably valid. The difference is that we get a slight increase. We believe this may go up to 30 percent if you measure it carefully. Thus, you may get 30-percent greater yields under simultaneous conditions.

We think the difference is in the rate of utilization of the organic compound you are working with. This was indicated by the work we did with the *H. facilis* mutant. Now that we have mutants of *H. eutropha*, we are going to do exactly the same thing.

We feel that if you have a readily utilizable organic compound, the organism is going to grow mainly heterotrophically until this compound is exhausted. Then the organism switches metabolism. If you have a slowly utilizable compound, the organism is going to grow simultaneously, presumably saturating, the electron transport or some other system. I believe this is the difference.

Most of the people who did the early work on these organisms used lactate or acetate. These compounds are not utilizable nearly as rapidly by *H. eutropha* or *H. facilis*, as are amino acids and carbohydrates to some extent. Even carbohydrates are utilized much more slowly.

We have done similar things with fructose. The generation time on glutamate is about one-half of what it is on fructose. And, as Bob Tischer said, fructose is the only carbohydrate of the ones that we have tested that *H. eutropha* normally can utilize.

Dr. REPASKE. You mentioned the glutamate generation time was about 2 hours in your experiments. What was the generation time for the autotroph?

Dr. DECICCO. Surprisingly, it was shorter than I thought it would be. It seemed to be a little over 3 hours once they got going. This was a little faster than I expected.

Dr. COX. I saw something on one table that indicated you did not get complete clearing. Is that true?

Dr. DECICCO. This is true. We do not get complete clearing. As a matter of fact, we begin to get repopulation before complete clearing.

Dr. COX. Could I have your thoughts on the possibility that this might be due to episomal factors of control? I presume these organisms believe in sex.

Dr. DeCicco. When you are working with one strain you have a problem.

Dr. Repaske. You mean all male or all female?

Dr. DeCicco. Presumably they are all male or all female, unless you get variants thrown off. Then you are getting these $F+$ and $F-$ variants occurring in the same culture.

I do not see that the episomal factor figures into the clearing. This may very well be a factor in some things.

You have a lot of problems of this type when you are dealing with a single strain of one organism. The same problems arise with *H. facilis*. *H. facilis* was isolated once, and this same strain, as far as I know, is being used by everybody. You have these same problems unless you have this mutation from an $F-$ to an $F+$, or vice-versa, whichever the original strain was. You are going to have essentially one type of organism.

When you are talking about sexual processes, these are usually done with *Escherichia coli* or two different strains derived from two different animals presumably at two different times, and possibly in two different countries. Here, with the single strain, I do not know if the situation would be the same.

As I mentioned, under heterotrophic conditions you may get essentially complete clearing. You may never get complete clearing because of cell debris which may give you some turbidity. It depends on, again, the multiplicty of infection and what the particular organic compound may be. If you use a simple medium like glutamate salts, you are not going to get as much activity as if you used trypticase soy agar or some richer medium.

Dr. Repaske. And yet on plates you do not get complete clearing?

Dr. DeCicco. Yes, you do. Heterotrophically. As I mentioned, this is how we get the phage-resistant cultures. You put enough phage in the plate to get complete lysis. In 18 hours you will see a completely clear plate. And then within a few more hours you will start to see resistant colonies developing. By the next day, after 36 hours, these will be quite low.

Dr. McFadden. In those checks for sensitivity during autotrophic growth to the phage, what concentration of trypticase soy broth or agar would you use? Very low, I presume.

Dr. DeCicco. Half of the recommended concentration. The recommended one is 30 grams per liter. We usually use 12 to 15. So here we would be dealing with 15 grams per liter of trypticase soy broth, and about half of that is amino acid. You have a little phosphate and a little glucose.

Dr. McFadden. Is it possible that the organisms are using that preferentially?

Dr. DeCicco. Yes, it is; except I do not think there was enough there for them to utilize only that. It would be too limited. Again, if you are dealing with 15 grams per liter, the utilizable organic compounds would be probably about 5, because about half of the 15 grams is amino acid, and half of the amino acid is utilizable. You're down to one-fourth of 15 grams, which is 4 grams per liter. Then you are using 1-percent inoculom. This is giving you 40 milligrams per liter; it isn't a large amount of nutrient.

Dr. Krauss. You showed the resistance to phage of organic media. It is possible that there is a quite different rate of movement for resistance in an inorganic medium?

Dr. DeCicco. Right. It may be that the bacteria are more resistant. It may be that you are getting lysogeny. We don't know exactly.

With a lot of other phages, when you slow down the growth rate, the phages just aren't as effective. I don't know exactly why it is, but there is a decreased activity under, again, strictly autotrophic conditions. There is also decreased activity under a simpler medium, a less complex medium, such as glutamate salts or fructose salts or something like this.

Dr. Repaske. Is any phage sensitive to destruction in a medium of improper tonicity?

Dr. DeCicco. It depends on how improper; but normally, no. Are you referring to hypertonic?

Dr. Repaske. Yes.

Dr. DeCicco. I will qualify that. Phages will survive a lot longer in amino acid or peptone or protein-containing media. If you put them in a salts medium, you get a loss of titer fairly rapidly. At one time I resuspended them in an inorganic medium. One or two weeks later I checked it, and it was of very, very low titer. So, these do lose infectivity again.

Dr. Repaske. Could this be the difference between a heterotrophic medium and autotrophic?

Dr. DeCicco. I would not think so. You would expect the phage to attach and begin infection within a matter of hours under any conditions, including autotrophic conditions. I would not expect that you are getting much occurring in this short period of time, as far as loss of titer goes.

Dr. Repaske. But that is another reaction, isn't it?

Dr. DeCicco. But again, your salts may play a role. Certain salts are required for attachment; certain materials inhibit attachment. This would all have to be looked at. Usually, magnesium is required for attachment. You would expect this would make the autotrophic medium more effective. We do not know whether it plays a role with these particular phages or not. Some phages require organic compounds for infectivity. I know there is a tryptophane-requiring phage; I think it's an *E. coli* phage. But, we do not know anything about these.

Dr. Cox. This might have to do with the synthesis of particular acceptor sites.

Dr. DeCicco. Certainly the resistance we would expect, if the clones that develop are truly resistant as opposed to being lysogenic (another form of resistance), that they would have altered receptive sites.

A lysogenic phage, that is a bacterium which is carrying a pro-phage may be resistant to subsequent infection. It would be harboring phage nucleic acid and would not have presumably altered receptor sites.

Dr. Cox. No, I am not referring to the synthesis of more mature receptor sites.

Dr. DeCicco. Actually you're talking about a difference in the cell-wall structure under both conditions.

Nutritional Properties of Harvested *Hydrogenomonas Eutropha*

Doris H. Calloway
University of California, Berkeley
Berkeley, California

Studies of the hydrogen-fixing bacterium, *Hydrogenomonas eutropha*, indicate that this organism may form the basis of a bioregenerative system for atmospheric control during prolonged space flight. Although this development would permit great savings of weight and power in comparison with complete storage systems and even with the algal system proposed earlier, maximum benefit can be gained only if the harvested cells can serve wholly or partly as food for the crew. Brief studies, using small amounts of dried organism made available by other investigators, demonstrate the bacterial protein to be readily digestible and of high biological quality, whereas the majority of the unprocessed lipid is not available.

COMPOSITION

Samples received from the Battelle Memorial Institute (BMI) and from Mississippi State University (MSU) were harvested by centrifugation from adequate media during log-phase growth. The Research Institute for Advanced Studies (RIAS) has provided samples harvested under conditions of rapid growth and after a period of exposure to a growth-limiting medium low in nitrogen content. The composition of the cells, given in table I, includes about 13 to 14 percent of nitrogen on a dry-solids basis irrespective of source, except when the medium is deficient. Cells from deficient medium have an increased proportion of fat, 23 percent instead of about 9 percent, as in the samples from rapidly growing cultures. Residual medium, harvested and dried with the cells, contributes substantial amounts of ash constituents and some nonprotein nitrogen; five washings in distilled water were needed to eliminate completely the nonprotein nitrogen from the supernatant fluid.

Mineral content varies widely, apparently because of differences in composition of media and of the amount harvested with cells. Potassium ranges from 0.3 to 9.0 mg per gram of dry solids, calcium from 0.1 to 2.1 mg, and magnesium from 0.2 to 2.0 mg. Since the orga-

Table I.—*Composition of* Hydrogenomonas Eutropha *Cells*

Constituent	Source			
	BMI[a]	MSU	RIAS	
	g per 100g dry solids[b] (4 samples)	g per 100g dry solids (2 samples)	g per 100g dry solids	
			(1 sample)	(2 samples)
Nitrogen	12.90–14.700	12.70–14.20	(13.1)	(8.1) 8.90
Lipid	7.20– 9.400			23.00
Ash	2.400	5.50– 5.70		
Phosphorus	0.820	1.40– 1.80		
Chloride	0.074			
Calcium	0.02– 0.210	0.20		0.01
Magnesium	0.02– 0.100	0.20		0.04
Potassium	0.03– 0.620	0.91		0.79

[a]BMI cells washed free of medium; MSU and RIAS cells were not washed. Unwashed BMI cells contained 5.9 percent of ash and 1.7 percent of phosphorous.
[b]Dried cells contained 6 to 9 percent of water as received. Data have been converted to a moisture-free basis for comparability except for ^{14}C-labeled RIAS samples shown in parentheses which are "as is."

nism has no stated requirement for either potassium or calcium, these figures seem to be adventitious.

The amino-acid composition of *H. eutropha* protein published by Foster and Litchfield (ref. 1) indicated serious deficiencies in several essential amino acids but accounted for only 64 percent of the nitrogen (assuming the protein to have a conventional 16 percent of nitrogen). Our data account for 96 percent of the nitrogen and correspond almost exactly with the BMI pattern at 1.5-times concentration (table II). The only significant difference is in lysine content, where our higher value may be due to a more gentle drying procedure. Amino-acid content and pattern place the bacterial protein in a class with the milk protein—casein—and with muscle meat and soy beans. A single BMI sample analyzed by Dr. David Schwarz (Schwarz Bioresearch, Inc.) contained 7.8 percent of nucleic acids. The ratio of DNA to RNA is unusual: 0.3 of DNA to 7.5 of RNA, i.e., 1:25. Total nucleic acid is also well below that of *Escherichia coli* (13 percent).

Soxhlet extraction of high-protein BMI cells with diethyl ether removes only 0.45g of lipid containing the following fatty acids: C12:0, 14:0, 16:0, 16:1, 17:0, 18:0, 18:1 and 18:2. If mixed-solvent systems are used (either chloroform-methanol or diethyl and petroleum ethers with ethanol), 6.10g of lipid are extracted and, in the latter case, 9.39g if extraction is preceded by hydrolysis of mild hydrochloric acid. The major part of this lipid is presumed to be beta-hydroxybutyric acid ($CH_3CHOHCH_2COOH$) or a polymer thereof, but the extract contains some digitonide-precipitable sterol and alpha-tocopherol (80 and 5.76 mg per 100g dry BMI cells, respectively). Neither vitamin A nor carotenoids are present.

Two samples of RIAS cells containing only 8 percent of nitrogen had about 23 percent of lipid, extractable after acid-hydrolysis with a mixed-solvent system. These samples must be re-evaluated under new extraction conditions if we are to account for 100 percent of cell dry weight. The residuum (not yet determined) could be carbohydrate but, as none is present in high-protein cells, the lipids may not have been completely removed.

TABLE II.—*Amino Acid Composition*

Amino acid g/16g nitrogen	Whole egg (ref. 2)	Casein (ref. 2)	*Hydrogenomonas eutropha* sterile autotroph			*Chlorella pyrenoidosa*	
			Battell	Analysis	Univ. Calif BMI sample	Sterile autotroph (ref. 3)	Sterile heterotroph (ref. 3)
			as published (ref. 1)	X1.5			
Tryptophan	1.65	1.34	1.05	1.6	N.A.	----------	----------
Threonine	4.98	4.30	2.90	4.4	4.52	8.02	7.84
Lysine	6.40	8.06	3.57	5.3	8.61	6.72	8.30
Methionine	3.14	3.10	1.54	2.3	2.69	1.82	2.05
Cystine	2.34	0.38	0.11	0.2	----------	----------	----------
Isoleucine	6.64	6.59	2.92	4.4	4.58	2.52	2.84
Leucine	8.80	10.11	5.44	8.2	8.52	7.26	7.49
Phenylalanine	5.78	5.42	2.96	4.4	3.96	4.43	4.37
Tyrosine	4.30	5.86	2.41	3.6	3.26	2.91	3.06
Valine	7.42	7.44	4.08	6.1	7.13	4.04	4.72
Histidine	2.40	3.04	1.28	1.9	2.48	1.41	1.69
Arginine	6.56	4.10	4.59	6.9	8.00	5.15	5.88
Alanine	(7)	3.38	6.02	9.0	8.80	9.08	9.59
Aspartic acid	7.01	7.44	5.82	8.7	9.57	5.61	5.64
Glutamic acid	1.24	2.32	10.33	15.4	11.17	10.73	11.48
Glycine	3.54	2.00	3.72	5.6	5.47	5.30	5.23
Proline	4.24	11.82	2.77	4.2	3.46	3.54	3.68
Serine	8.40	6.69	2.42	3.6	3.47	3.44	3.84
Total	91.84	93.39	63.93	----------	95.69	81.98	87.70

BIOLOGICAL QUALITY OF PROTEIN

Although the ability of *H. eutropha* to grow in the intestinal tract is uncertain, the conditions appear favorable since gaseous hydrogen, oxygen and carbon dioxide are present, nutrient medium is available, and the temperature is within the organism tolerance range. Therefore, a number of methods of disintegrating the cells, preferably without heat, were tried in order to prepare a non-viable sample suitable for animal-feeding studies. Most of these methods were unsuccessful but ultimately two methods were accepted: heating for 15 minutes at low boiling temperature in aqueous suspension, and exposure to high-frequency sound. This latter process ruptured only about 60 percent of cells initially but fragmentation was nearly complete when the cells were stood in cold aqueous suspension (4°C) for 24 hours.

Biological quality and digestibility of protein of BMI no. 1 were determined by an abbreviated Mitchell-Thomas method. A diet containing 4 percent of protein from dry whole egg was fed for 1 week to 20 weanling male albino rats (Simonsen-strain, cesarean-derived from Sprague-Dawley stock). These were assigned on a matched-weight basis to four dietary treatment groups: boiled bacteria, sonified bacteria, casein, and 4-percent egg protein. Bacteria and casein were incorporated into diets at levels to yield 1.6 percent of nitrogen on a dry-solids basis (table III). As used, the diets contained 40 percent of moisture. Because of the limited amount of bacteria available, the test diets were fed for only 1 week; urine, feces, and rejected food were collected quantitatively for the last 5 days. These samples and all diets were analyzed for nitrogen content (AOAC, 1960). Biological value listed in table IV, was computed according to the method of Mitchell et al. (ref. 4) using the following factors:

NI: nitrogen intake, mg

FN_m: fecal nitrogen of the 4-percent protein group (metabolic)=0.945 mg per gram of dry food eaten

FN_t: fecal nitrogen of the test group, mg

UN_e: urinary nitrogen of the 4-percent protein group (endogenous=0.360 mg per gram 0.75 of body weight per day)

UN_t: urinary nitrogen of the test group, mg

Absorbed nitrogen (*AN*): $NI-(FN_t-FN_m)$

Nitrogen digestibility (*ND*), percent: $\frac{AN}{NI}\times 100$

Biological value (*BV*), percent:

$$\frac{A_tN-(UN_t-UN_e)}{AN}\times 100$$

Net nitrogen utilization (*NNU*), percent: $ND\times BV\div 100$.

The digestibility (or, more properly, net absorption) of *H. eutropha* nitrogen was 93 as compared with that of casein, 99 percent, but was within the normal range and on a par with that of mixed human diets, cereals and cooked legumes. Retention of absorbed nitrogen (the biological value of 77 percent) indicates a well balanced pattern of amino acids, as good as that of casein. Using this same general method, biological value of whole-egg protein is normally about 97 percent; of fresh whole milk, 90 percent, of fresh muscle meats, 74–78 percent, and of casein about 75 percent.

LIPID DIGESTIBILITY

Digestibility of the lipid and protein found in RIAS high-fat cells and that of BMI high-protein cells was measured by incorporating boiled cells in diets fed to mice. To obtain values for metabolic lipid and nitrogen, one group of mice was fed a diet very low in protein and fat. Control animals were fed a diet based on corn oil and casein (table V). Diets were fed to groups of six or seven male albino mice (Carworth Farms) for 10 days. Food intake was measured and feces were quantitatively collected during the last 5 days. Both diets and feces were analyzed for lipid content by extraction with mixed ethers and alcohol after mild acid hydrolysis; nitrogen was measured by micro-Kjeldahl analysis. Digestibility was computed as indicated for protein above. In order to ensure adequate absorption of fat-soluble vitamins and a supply of essential fatty acids, all diets contained 1 percent of corn oil. The RIAS diet contained 10 percent of bacterial lipid, and the BMI provided 3 percent, within the total 11 percent of lipid in all diets. Protein composition was determined by the RIAS sample: this was 25 percent of all diets.

From the control diet, 99 percent of both lipid and protein were absorbed (table VI—values given are means of 6 mice per group). With the RIAS cells, digestibility was only 19 percent of total lipid; since 10 percent of the lipid was corn oil (99-percent absorbable), the digestibility of bacterial lipid is not more than 10 percent. The lipid contained in the BMI diets was 47-percent absorbed, or less than the amount of corn oil present. Nitrogen digestibility was 93 percent with the RIAS sample and 94 percent with the BMI, in good agreement with rat

TABLE III.—*Diets*

Components	Egg 4-percent protein	Casein standard	*H. eutropha* (boiled or sonified)
Whole egg (dry)	8. 6		
Casein, vitamin-free [a]		12. 0	
H. eutropha, #B1 (dry)			12. 0
Cornstarch	70. 0	64. 4	64. 4
Sucrose	9. 0	9. 0	9. 0
Cottonseed oil	4. 4	6. 6	6. 6
Alpha-cellulose	2. 0	2. 0	2. 0
B-vitamin mixture [a]	1. 0	1. 0	1. 0
Salts USP XIV + zinc [b]	5. 0	5. 0	5. 0

[a] The sucrose mixture provided, in mg per 100g diet: thiamine HCl, 0.25; riboflavin, 0.50; pyridoxine HCl, 0.25; niacin, 2.0; Ca pantothenate, 2.0; inositol, 10.0; para-aminobenzoic acid, 5.0; biotin, 0.01; folic acid, 0.10; vitamin B_{12}, 0.005; and choline Cl, 100.0. Fat-soluble vitamins were given twice weekly by dropper, providing per rat per week: vitamin A, 600 I.U.; vitamin D_2, 10 I.U.; alpha-tocopherol, 3.0 mg; and 2-methyl-1,4-naphthoquinone, 0.3 mg.

[b] Two parts of zinc carbonate added to 598 parts of USP XIV salts mixture, which is adequate for the rat with respect to minerals other than zinc.

TABLE IV.—*Protein Quality of Nitrogen of* H. eutropha

Sample [a]	Biological value (percent)	Digestibility [b] (percent)	Net utilization [b] (percent)
H. eutropha:			
Boiled	77. 1	93. 0	72
Sonified	76. 0	93. 6	71
Casein	77. 3	98. 9	76

[a] Fed as the sole source of protein in diets containing 1.6 percent of nitrogen on a dry-solids basis.

[b] Means of five rats per group.

TABLE V.—*Diets Fed to Mice for Digestibility Trials*

Component	RIAS	BMI g/100g diet	Control
Lipid-rich RIAS *H. eutropha*	43. 5		
Protein-rich BMI *H. eutropha*		30. 0	
Casein			30. 0
Corn oil		6. 0	10. 0
Salts USP XIV plus $ZnCO_3$ [a]	6. 0	6. 0	6. 0
B-vitamins mixture [a]	1. 0	1. 0	1. 0
Fat-soluble vitamins in corn oil [b]	1. 0	1. 0	1. 0
Cornstarch	24. 25	28. 0	26. 0
Glucose	24. 25	28. 0	26. 0

[a] See table III.

[b] The corn-oil mixture provided, per 100g of diet: 1776 *IU* vitamin A as palmitate, 160 *IU* vitamin D_3, 1.82 mg oα-tocopherol, and 0.20 mg vitamin K_3.

TABLE VI.—*Digestibility of* Hydrogenomonas eutropha *Lipid*

	RIAS lipid-rich	BMI protein-rich	Casein corn oil
Food eaten, g/mouse/5 days	27	30	34
Lipid digestibility, percent	19	47	99
Nitrogen digestibility, percent	93	94	99

data for the comparable BMI sample (see above).

Good protein digestibility in spite of poor fat digestion (indicating that the high-fat cells are not resistant to digestion) suggests that the lipid is located within the cells and that the cell membrane is not made more resistant by the cultural conditions. There is no evidence that the cells have toxic properties in the brief test period; nevertheless, the diet was less well accepted by the mice than that based on the BMI cells or casein.

COMMENT

Reasonable caloric intake for man, based on organisms of this composition, would require consumption of about 700–800g of bacterial solids daily, providing about 90–100g of nitrogen and 50–60g of nucleic acids if the digestible high-protein variant were used. At conventional renal concentration, clearance of this amount of nitrogen as urea would require excretion of 4–5 liters of urine. If the purine and pyrimidine bases are absorbed, the large quantities converted to uric acid (the end-product of purine metabolism) could present serious problems. Since uric acid is relatively insoluble, there would be substantial risk of forming urate stones in the renal system or development of gout. Animals do not readily accept or thrive on diets containing more than 40 to 50 percent of protein. All these factors indicate that high-protein organisms cannot serve as the sole dietary energy source. The question whether the bacterial lipid can be processed into an available and non-toxic form must be answered affirmatively, or the bacterial production must be supplemented with other food sources.

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COMMENTS

Dr. GOLDNER. How far are you willing to extrapolate to man the data you obtained in mice as to lipid digestibility?

Dr. CALLOWAY. If the mice do anything that we don't do, they recycle it probably. If anything, it has a double set of opportunities to be digested in mice. We do not know that there is any difference in lipolytic activity in the human gut and in the mouse gut. We normally see 98- to 99-percent digestibility of conventional lipids in both man and the rodents we used as experimentals. I think the chances are not very good that the lipid will be used as such. You may have to isolate the depolymerase that presumably is contained in the organisms itself, and use that to process your material. In its present form, there is very limited hope of its use.

Dr. KRAUSS. I really don't believe it is at all realistic to think in terms of feeding whole cells of any sort as a diet. You have to include processing the cell masses in this program. This should be clear to everybody by this time.

Dr. CALLOWAY. I think so. Perhaps a two-component system has a lot to be said for it, too. This simple-minded approach of "one man and one bug," without a lot of something in the middle, has got to cease. It is going to cost us at least as much as a rocket costs to figure out whether we can send him with any one of these.

Dr. JENKINS. Is there any toxicologic reaction at all from this lipid-like material?

Dr. CALLOWAY. Nothing. It all came out apparently as though it were barium sulphate or anything else. It apparently carried out a little of the lipid along with it. But that would happen normally. Anything that would give you that amount of residue is likely to take on additional water, salts and other things. It would be my guess that the amount that is extractable with conventional sohxlet extraction appeared to be the percentage that could be digested.

Dr. DECICCO. Is there any other evidence that you or anyone else knows that the poly betahydroxybutyric is digested?

Dr. CALLOWAY. As far as I know, no one else has tried it except us.

PHBA showed us no reason why lipid should not be digested. But it was not to be. We made some esters of long-chain fatty acids and long chain alcohols that have the conventional ester bond. They would not split in the gut, either.

Growth of *Hydrogenomonas Eutropha* in Urine for Application to a Bioregenerative Life-Support System

BERNARD H. GOLDNER AND J. B. DITTMAN
TRW, Inc. Systems
Redondo Beach, California

In closed ecological systems proposed for extended manned missions, it will be necessary to regenerate food from human wastes, such as CO_2, urine, and feces. In addition, a suitable atmospheric balance between C_2 and CO_2 must be maintained. A bioregenerative life-support system that offers the advantages of producing food material from wastes, as well as providing an atmospheric balance, is being developed and consists of combining the electrolysis of water with the autotrophic growth of *H. eutropha.**

Urine can serve as a major source of nutrients for *H. eutropha* since it contains urea as a nitrogen source and all the inorganic salts required in the autotrophic nutrition of this bacteria. This paper describes an investigation of the nutrition, physiology, and culture characteristics of *H. eutropha* grown in urine, with the result that a material balance is feasible between the volume of urine excreted, the amount of CO_2 produced by human respiration, and the volume of CO_2 utilized by a culture of *H. eutropha.*

MATERIALS AND METHODS

Urine

The urine, furnished by D. Calloway (University of California, Berkeley), was collected from four healthy male subjects who were placed on a nutritionally adequate formula diet containing 2800 calories per day for 6 weeks. Urine collected during the final 3 days of the diet study was used for this program. The total daily output, which averaged 1500 ml, was diluted to 2 l with deionized water; thus, the urine as received was diluted 25 percent. The total amount of urine collected was pooled, frozen in small lots, and maintained at −20° C. The urine was quick-thawed for growth experiments and sterilized by filtration through a 0.22-μ membrane filter (Millipore Corp., Bedford, Mass.). Filter-sterilized $Fe(NH_4)_2(SO_4)_26H_2O$ was added routinely to a final concentration of 11 mg/l.

Culture Apparatus

The growth vessel (fig. 1) consisted of a 500-ml baffled Erlenmeyer flask provided with two sampling ports. One port was fitted with a rubber septum to permit syringe sampling of either the culture or gas phase. A second port allowed the insertion of a combination pH electrode into the culture. Some growth vessels were modified with a third port for the insertion of a Beckman oxygen-macro electrode. The culture vessel was fitted with a head assembly that contained a stainless-steel impeller driven by a variable-speed motor and a stainless-steel tube that delivered the gas mixture directly into the vortex created by the stirring motion of the impeller. The head assembly also contained a gas outlet

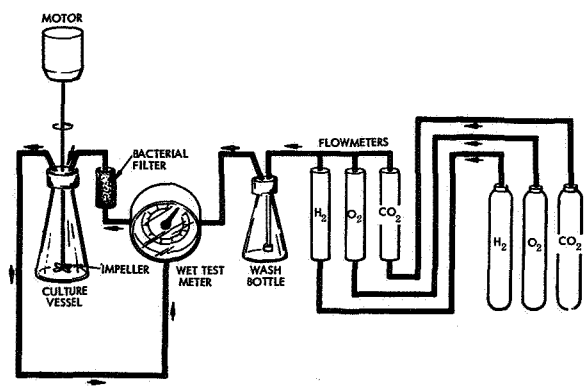

FIGURE 1.—Flow diagram of respirometer used to measure growth and gas uptake of H. eutropha cultured in urine.

H. cutropha obtained from R. Repaske (National Institutes of Health, Bethesda, Maryland) was used throughout the study.

port. The gases (H_2, O_2, and CO_2) contained in individual gas cylinders were proportioned by individual flowmeters and mixed in a gas manifold. The gas mixture was humidified to reduce evaporative losses in the culture by sparging in a wash bottle. Sterilization of the gas mixture was accomplished by passage through a glass cylinder packed with glass wool. The culture assembly was sterilized by autoclaving. The pH electrode and oxygen sensor were sterilized by overnight immersion in Roccal solution.

The rate of gas consumed by the culture was determined with a calibrated wet-test meter, which alternatively measured the inlet and outlet gas flow by a manual valve-switching device. An average deviation of 0.4 l total gas/hr existed between the inlet and outlet gas-flow rate. The rate of the total amount of gas consumed by the culture was measured at hourly intervals.

In view of the requirement to produce a balance between the rate of CO_2 respired by man and the rate of CO_2 fixed by *H. eutropha*, the maximum rate of gas uptake was considered to be the most significant parameter of culture performance and, therefore, conditions were optimized for gas uptake rather than growth, per se.

The gas mixture consisted of 8.3 percent O_2, 10 percent CO_2, and 81.7 percent H_2; the impeller stirring speed was controlled at 2000 rpm unless indicated otherwise. A gas-flow rate was maintained at 22 l/hr. Experiments were carried out at 35° C by placing the entire assembly in a constant-temperature incubator.

Preparation of Culture

To prepare *H. eutropha* for the measurement of gas uptake and growth in the culture apparatus, the culture was grown with shaking at 30° C in 500-ml glass-stoppered Erlenmeyer flasks containing 25 ml of the experimental medium. The flasks were gassed with a mixture consisting of 70 percent H_2, 20 percent O_2, and 10 percent CO_2 by placing the cotton-plugged flasks in an anaerobic jar, alternately evacuated and filled with the gas mixture, and finally replacing the cotton with glass stoppers when gas equilibrium was reached.

After a 16- to 18-hour growth period, the cells were centrifuged at 5° C and washed once with sterile physiological saline. The cells were then resuspended in 50 ml of the experimental medium to obtain an optical density of 6.0 to 7.0 as measured at 660 mμ in a Beckman DB spectrophotometer. Just before transfer of the suspension to the growth vessel, freshly prepared $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was added and the pH adjusted between 6.8 and 7.0 with 0.1 N NaOH. In most cases the same medium was used in the shake-flasks and culture apparatus to ensure adaptation of the culture to the experimental medium. Culture purity was checked routinely by microscopic examination and by streaking agar plates prepared with trypticase soy agar (BBL).

Analytical Methods

Urea was determined by the biacetyl monoxime method (ref. 1). Trace metals were analyzed semiquantitatively by emission spectrographic analysis. Amino acids were determined with a Technicon column chromatograph, and total nitrogen was determined by the Kjeldahl method. Poly-beta-hydroxybutyrate was analyzed according to the procedure of Williamson and Wilkinson (ref. 2). Potassium and sodium were determined by flame photometry. The procedures used for the remaining urinary inorganic ions and organic compounds (table I) are described by Henry (ref. 3). Dry weight of the cells was determined by washing the cells twice with distilled water and drying to a constant weight under vacuum at 50° C.

RESULTS AND DISCUSSION

Composition of Urine

Aliquots of urine were quick-thawed, sterilized by membrane filtration, and analyzed for inorganic and organic components and trace metals. Normal urine contains over 200 constituents and, therefore, the urine was analyzed primarily for those constituents reported to be present at relatively high levels and known to be involved in the nutrition of bacteria.

Table I shows that the inorganic composition of the urine falls within the range reported for normal urine (ref. 4), but that the concentration

TABLE I.—*Composition of Urine*

Inorganic:	*mg/l*
Magnesium	75. 6
Phosphorus	370. 0
Sodium	1170. 0
Potassium	980. 0
Calcium	56. 1
Sulfate	420. 0
Chloride	2230. 0
Trace metals:	*ppm*
Copper	1. 7
Silicon	24. 0
Boron	0. 65
Aluminum	2. 5
Iron	1. 5
Molybdenum	0. 14
Organic:	
Urea	6665. 0
Glucose	20. 0
Uric acid	210. 0
Lactic acid	40. 0
Citric acid	700. 0
Creatinine	400. 0
Hippuric acid	0. 0
Amino acids:	*μM/l*
Alanine	90. 0
Glycine	839. 0
Lysine	57. 0
Methionine sulfoxide	132. 0
Phenylalanine	27. 0
Serine	255. 0
Taurine	296. 0
Tyrosine	28. 0
Histidine and 3-methylhistidine	306. 0
Threonine, asparagine, and glutamine	80. 0
Nitrogen:	
Total N	3900. 0
Alpha amino acid N	73. 0
Ammonia N	10. 0

of organic nitrogenous compounds, particularly urea, is low. The diet of the urine donors contained a normal amount of protein (75 g/day), but it is possible that protein assimilation was increased by the type of diet employed in the study, thereby accounting for the low content of nitrogenous compounds in the urine.

Dilution of Urine

Studies carried out in synthetic media have fairly well defined the minimum nutritional requirements for the autotrophic growth of *H. eutropha* (ref. 5). In addition to H_2, O_2, and CO_2, the bacteria can be cultivated successfully in a medium containing urea, magnesium, phosphorus (as phosphate), sulfate, potassium, ferrous iron, and various trace metals (not yet defined). Table II shows that undiluted urine contains levels of three essential nutrients (urea, magnesium, and sulfate) considerably higher than Bongers' medium, which is considered to be fairly optimum for autotrophic growth. The potassium and phosphorus content of urine and Bongers' medium cannot be strictly compared because potassium phosphate is used as a buffer in Bongers' medium and the molarity varies according to experimental conditions. Urine is deficient in ferrous iron; consequently, the urine must be supplemented with Fe $(NH_4)_2(SO_4)_2 \cdot 6H_2O$.

TABLE II.—*Comparison of Undiluted Urine with Bongers' Medium*

	Undiluted Urine (mg/l)	Bongers' Medium[a] (mg/l)
Urea	6650. 0	1000. 0
Magnesium	75. 6	9. 85
Phosphorus	370. 0	759. 2
Potassium	980. 0	1543. 2
Sulfate	420. 0	39. 0

[a] 0.025 M, Sorensen's potassium phosphate buffer (pH 7.0).

The excess nutrients contained in urine indicated that the urine could be diluted with water to achieve a balance between the volume of urine excreted in 24 hours (approximately 1.5 l) and the volume of CO_2 produced by human respiration (22 l/hr.) The amount of CO_2 uptake by a culture of *H. eutropha* is partly a function of the density of the cell suspension and the volume of urine available. Since the urine appeared to contain more nutrients than required to support optimum growth, it was considered more practical to dilute the urine to increase the volume of culture rather than to attempt to achieve extremely dense cultures in undiluted urine.

Figure 2 shows the results of diluting the urine with distilled water to a final concentration ranging from 5 to 50 percent of undiluted urine (as received). The maximum rate of gas uptake occurred in 50 percent urine (26.0 l total

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gas l culture/hr) a 6.6-percent improvement over that obtained in undiluted urine. This may be attributable to either a reduction in concentration of some inhibitory urine component or to dilution of the urine to a more favorable ionic strength. Gas uptake rates in 25 and 15 percent urine were similar but, in 5 percent urine, gas uptake was considerably reduced.

Table III shows the relationship between dilution of the urine and the corresponding decrease in the rate of maximum gas uptake. It is evident that the most favorable ratio between dilution, gas uptake, and volume occurred in 15 percent urine; a large culture volume was obtained which could still maintain a gas uptake rate that was 81.1 percent of undiluted urine.

TABLE III.—*Relationship Between Dilution of Urine and Gas Uptake*

Urine concentration (%)	Gas uptake of undiluted urine (%)	Maximum gas uptake rate (l total gas/l culture/hr)
Undiluted (75) [a]	100. 0	24. 4
50 (37. 5)	106. 0	26. 0
25 (18.75)	80. 7	19. 7
15 (11. 25)	81. 1	19. 8
5 (3. 75)	41. 0	10. 0

[a] Actual concentration of urine as received.

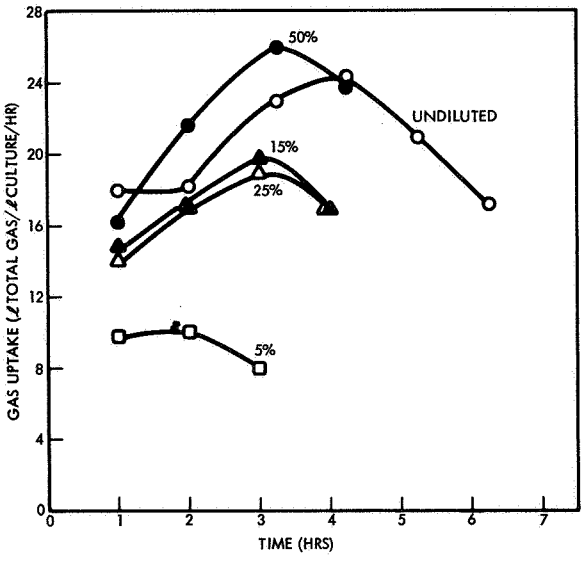

FIGURE 2.—Effect of dilution on gas uptake in urine.

Effect of Oxygen Concentration on Gas Uptake

The sensitivity of *H. eutropha* to O_2 is well documented (refs. 5 and 6). Inhibition of growth has been found to occur either in the absense of sufficient O_2 or in the presence of high concentrations of O_2. Oxygen concentrations below optimum lead to the formation of the storage product poly-beta-hydroxybutyrate, while high O_2 levels inhibit hydrogenase activity.

With the culture apparatus used in these experiments, the partial pressure of O_2 in the gas phase, the rate of gas flow, and the stirring speed of the impeller influence the concentration of dissolved O_2 in the culture. Consequently, experiments were undertaken to examine the influence of several of these variables on gas uptake rates. Early studies were performed in undiluted urine but were later carried out in 15 percent urine. With undiluted urine it was found that the best rate of gas uptake occurred with 8.3 percent O_2 in the gas phase (10 percent CO_2 and 81.7 percent H_2), a gas-flow rate of 22 l/hr, and an impeller stirring speed of 2000 rpm (fig. 3). Inhibition was evident at the same concentration of O_2 and gas-flow rate when the stirring speed was reduced to 1750 rpm. When the O_2 concentration in the gas phase was increased to 11.8 percent (10 percent CO_2 and 78.2 percent H_2), at a stirring speed of 2000 rpm and

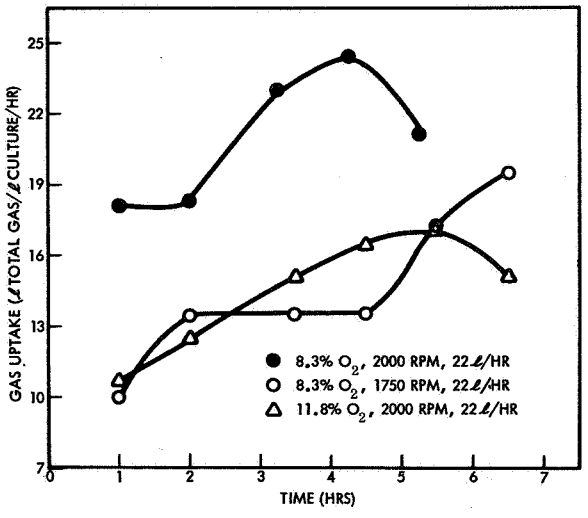

FIGURE 3.—Effect of O_2 concentration on gas uptake in undiluted urine.

a gas-flow rate of 22 l/hr, inhibition was also observed. The actual concentration of dissolved O_2 was not measured in these experiments, and it can only be assumed that O_2 reached equilibrium between the gas and liquid phases.

When 15 percent urine was adopted as the routine growth medium, a second set of studies was conducted. In these experiments, the concentration of dissolved O_2 in the cell suspension was measured with a Beckman oxygen-macro electrode. Figure 4 shows the influence of two concentrations of O_2, 8.3 and 9.9 percent, on gas uptake rates. In both cases the gas phase contained 10 percent CO_2 with hydrogen as the balance. The gas-flow rate was 22 l/hr and the impeller stirring speed was 2000 rpm. With 9.9 percent O_2 in the gas phase, the maximum rate of gas uptake was inhibited 15 percent, compared to that observed with 8.3 percent O_2. This essentially confirms the results obtained in undiluted urine. At both levels of O_2, equilibrium was reached initially between the gas and liquid phases; however, the dissolved O_2 concentration decreased slightly as growth progressed so that the final concentration of dissolved O_2 was reduced by 10 percent.

Influence of Contamination on Gas Uptake in Urine

Occasionally, urine cultures become contaminated despite rigorous precautions to maintain pure cultures. Figure 5 illustrates the inhibitory influence of a gram-positive bacillus and a gram-positive coccus on gas uptake in undiluted urine. In both cases, the maximum rate of gas uptake was inhibited 25 percent compared to the pure culture. The inhibitory effect cannot be attributed to unfavorable pH changes as the pH range of the contaminated cultures was similiar to the pure culture. Consequently, depressed gas uptake most likely resulted from either competition for nutrients or the production of toxic byproducts by the contaminants. No attempt was made to identify fully the contaminants or to enumerate their population relative to *H. eutropha*, but microscopic examination indicated that the majority of cells present at the end of the experiment was *H. eutropha*.

Contamination occurred with greater frequency when undiluted urine was used as the growth medium, but contamination has also been observed in 15 percent urine and even in strictly inorganic media. In view of the serious consequences of contamination in a life-support system, further study is required to understand the conditions under which bacterial contamination is likely to occur, the type of contaminants that might predominate, and the interactions of contaminants with *H. eutropha*.

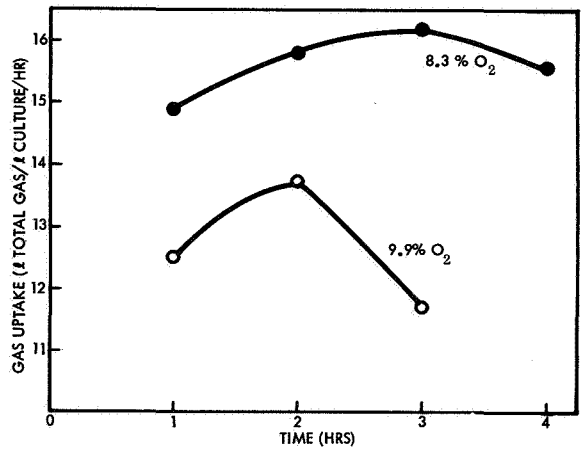

FIGURE 4.—Effect of O_2 concentration on gas uptake in 15 percent urine.

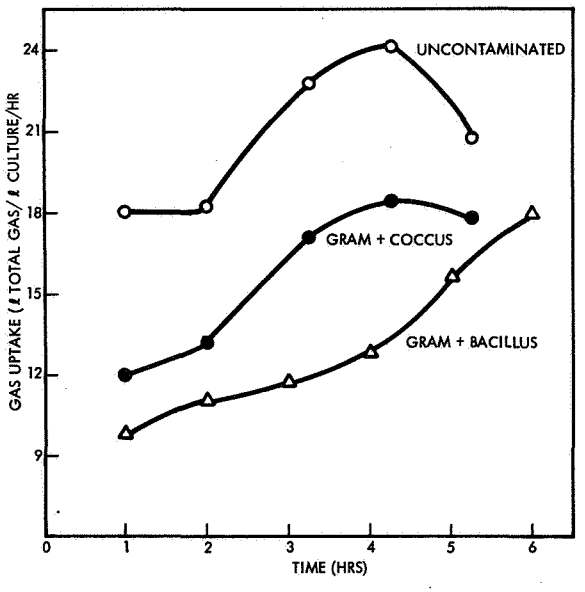

FIGURE 5.—Influence of bacterial contaminations on gas uptake in undiluted urine.

Addition of Nutrients to 15 Percent Urine

A series of experiments was undertaken to determine which nutrients were limiting growth in 15 percent urine in order to improve gas uptake rates since the 18.9-percent reduction in the maximum rate of gas uptake could probably be attributed to the exhaustion of an essential nutrient. The approach was to supplement 15 percent urine with each of three essential nutrients considered to be limiting—urea, magnesium (as magnesium sulfate), and phosphorus (as dibasic potassium phosphate), followed by various combinations of these nutrients. Sulfate and potassium were not studied as independent variables because 15 percent urine contained these ions in far higher concentrations than are normally contained in defined, autotrophic media. The amount of urea, magnesium, and phosphorus added to 15 percent urine was sufficient to increase the level to that contained in undiluted urine (see table I). The levels of each nutrient remaining in the culture after growth in the culture apparatus was analyzed after separating the cells from the growth medium by centrifugation (table IV). It should be noted, however, that the experimental periods were not the same in every experiment.

The results (table V) show that neither the addition of any single nutrient nor the combined addition of the nutrients to 15 percent urine improved the maximum rate of gas uptake. However, some interesting interactions were observed upon the addition of nutrients.

TABLE IV.—*Utilization of Nutrients in 15 Percent Urine*

Nutrient added	Urea utilized (%)	Magnesium utilized (%)	Phosphorus utilized (%)	Experimental period (hr)
None	95.4	61.0	>99.0	4
Magnesium	94.7	20.8	>99.0	4
Phosphate	95.4	73.2	31.4	4
Urea	44.3	41.7	100.0	6
Magnesium + phosphate	95.4	16.7	27.0	3
Magnesium + urea	45.0	24.4	>99.0	6
Phosphate + urea	38.7	91.0	38.8	5
Urea + magnesium + phosphate	50.0	25.0	46.9	7

TABLE V.—*Addition of Nutrients to 15 Percent Urine*

Nutrient added	Maximum rate of gas uptake (l total gas/l culture/hr)
None—undiluted urine	24.4
None—15% urine	18.4
Magnesium	18.8
Phosphate	15.2
Urea	18.8
Magnesium+phosphate	15.6
Magnesium+urea	18.2
Phosphate+urea	16.2
Urea+magnesium+phosphate	17.8

In three cases involving the addition of phosphate, inhibition of gas uptake was observed. When phosphate and urea were added to 15 percent urine, magnesium was virtually exhausted; this most likely contributed to inhibition of gas uptake. Inhibition occurred when phosphate alone, or phosphate and magnesium were added; however, an adequate level of both ions was maintained in the growth medium. The observed depressed gas uptake may have resulted from the inhibition of a phosphorylated intermediate by phosphate or may possibly be attributed to exhaustion of urea since no inhibition was evident when urine was supplemented with urea, or urea, magnesium, and phosphate.

The analytical determinations also show that, when urea was not added to urine, urea was virtually exhausted in every case. The same was true with phosphorus. In contrast, magnesium was severely depleted in only one case—when

the urine was supplemented with phosphate and urea.

Although supplementing urine with urea did not increase the maximum rate of gas uptake, figure 6 shows that the maximum gas-uptake rate was maintained at a steady state for a prolonged period while, in unsupplemented urine, gas uptake began to decrease immediately after the peak gas-uptake rate was reached. It is interesting that in urea-supplemented urine, despite steady-state gas uptake, phosphorus was not detected in the medium at the termination of growth. This raises the possibility that the cells store phosphorus intracellularly as polymetaphosphate.

Influence of Trace Metals on Gas Uptake in 15 Percent Urine

Because of the lack of response to major nutrients, attention was turned to the influence of trace metals on gas-uptake rates. Most inorganic media used for the culture of *H. eutropha* routinely contain a mixture of trace metals consisting of cobalt, manganese, copper, molybdenum, and zinc (ref. 7), although the precise nutritional requirements for these metals have not been established. Burris and Cohen (ref. 8) reported that the addition of trace metals to an inorganic media greatly stimulated the autotrophic growth of *H. facilis*. Bongers (ref. 5), however, found that with *H. eutropha* the omission of trace metals from a defined inorganic medium containing urea did not affect the rate of growth or gas uptake, and it was concluded that the reagent grade salts used to make up the medium contained an adequate amount of trace metals.

With 15 percent urine as the growth medium, supplemented with only a small quantity of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, the possibility existed that the medium was lacking in trace metals. To examine the nutritional role of trace metals, a series of experiments was performed in which the cells were washed and the urine diluted with a commercial grade of distilled water (redistilled once in a glass still) and varying quantities of Repaske's trace-metals solution (ref. 7) added to the growth medium. Figure 7 shows that, in comparison with the control lacking trace metals, the addition of the standard amount of trace metals ($1X$) increased the rate of gas uptake by 18 percent, while three times this amount enhanced gas uptake by 90 percent. The addition of $4X$ trace metals was inhibitory

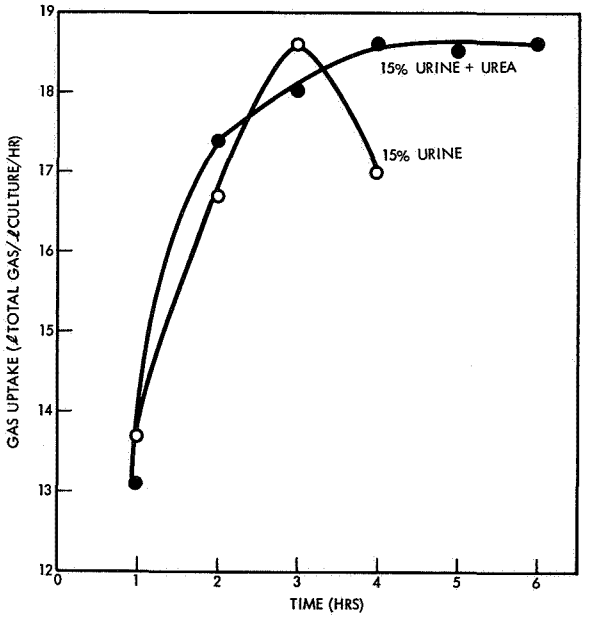

FIGURE 6.—Influence of addition of urea on gas uptake in 15 percent urine.

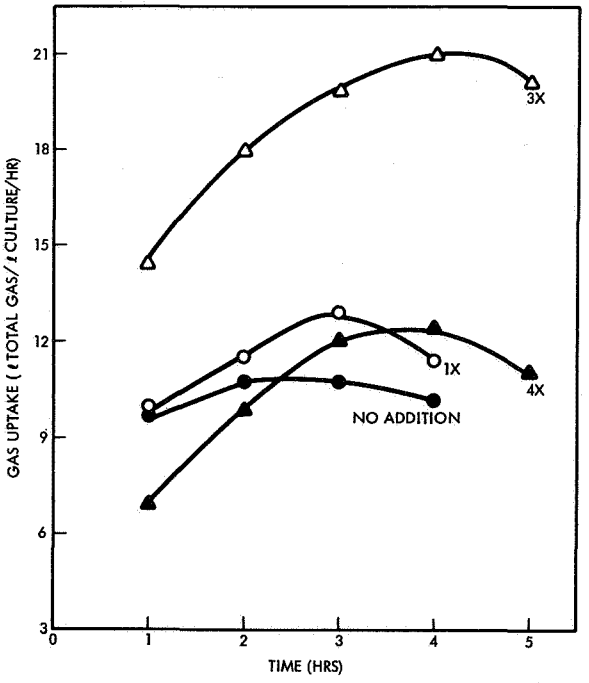

FIGURE 7.—Effect of trace elements on gas uptake in 15 percent urine.

and resulted in severely depressed gas uptake, compared to the urine containing $1X$ trace metals.

The maximum rate of gas uptake in urine containing $3X$ trace metals was only slightly higher than that observed in 15 percent urine diluted with a commercial grade of distilled water (fig. 2). However, redistillation of the diluent water resulted in far less gas uptake, suggesting that the commercial grade of distilled water contained considerable amounts of trace metals. When the distilled water was analyzed for trace metals, it was found to contain relatively high amounts of iron, boron, aluminum, zinc, silver, tin, sodium, calcium, silicon, in addition to 0.2–ppm copper. Redistilled water, on the other hand, contained only slight traces of sodium, copper, calcium, silicon, magnesium, and aluminum.

The level of copper in the distilled water suggested that gas uptake was being influenced by the copper since low levels of heavy metals can stimulate metabolism. Several experiments were performed in which it was shown that the addition of 0.2–ppm copper (as $CuCl_2$) to 15 percent urine diluted with redistilled water resulted in a 27-percent increase in the rate of gas uptake compared to controls lacking copper. Higher concentrations of copper were inhibitory. Therefore, the data indicate that the commercial brand of distilled water was influencing metabolism by providing essential trace metals and a stimulatory concentration of copper. As a result of these experiments, only redistilled water was used to dilute the urine, and trace metals were provided routinely by adding Repaske's trace-metals solution.

Influence of Organic Compounds on Gas Uptake in Urine

The ability of *H. eutropha* to metabolize heterotrophically and autotrophically at the same time prompted an investigation of the influence of organic urine components on gas uptake in an attempt to increase the rate of gas utilization and growth in 15 percent urine. Rather than attempt to remove all organic compounds from urine by chemical or physical means and risk altering the inorganic composition of urine, the approach was to formulate a "simulated urine" based on a quantitative analysis of all major inorganic constituents and urea (table I). Individual organic compounds could then be added to the simulated urine and their role more readily defined.

In the first experiment, gas-uptake rates in undiluted urine were compared to simulated urine (fig. 8), and it is evident that gas uptake was considerably poorer in simulated urine. The influence of glycine, the predominant amino acid in the urine, on gas uptake in undiluted simulated urine was examined in the next experiment. Glycine has been shown by DeCicco (ref. 9) to serve as a source of carbon and energy for *H. eutropha* without adaptation. The addition of glycine (0.395 mg/l) to the simulated urine resulted in a 40-percent increase in the maximum gas-uptake rate. The concentration of glycine selected was based on the alpha amino-acid N content in the urine.

Studies with glycine-supplemented urine were then carried out in 15 percent urine. Figure 9 shows that the addition of the same level of glycine to 15 percent urine increased the maximum gas-uptake rate by 52 percent, compared to the unsupplemented control. Cell yield only

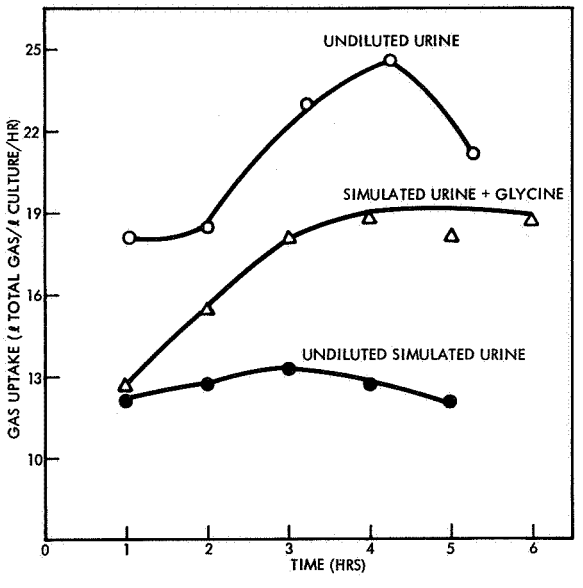

FIGURE 8.—Influence of glycine on gas uptake in simulated urine.

slightly increased by the addition of glycine—7.98 g/l compared to 7.46 g/l.

To determine whether glycine was stimulating hydrogenase activity or simply increasing the level of hydrogenase in the cells, an experiment was performed in which glycine was omitted from the shake-flask cells grown before resuspension in fresh medium. It was found that under these conditions, the addition of glycine did not result in enhanced gas-uptake rates, which suggests that glycine increased the level of hydrogenase in those cells that had been previously incubated in its presence. If the role of glycine was primarily to stimulate hydrogenase activity, then cells grown under autotrophic conditions in the absence of glycine should have responded similarly to the addition of glycine.

Culture Characteristics in 15 Percent Urine

Table VI summarizes the data obtained from a typical growth experiment performed in the culture apparatus. In terms of the maximum rate of gas uptake and cell yield, these experimental conditions are the best found thus far and involve the fewest nutrient additions to the medium. The medium consisted of 15 percent urine diluted with redistilled water supplemented with 11 mg/l Fe $(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 3X Repaske's trace-metals solution. Figure 10 illustrates gas uptake, optical density, and pH changes as observed under these conditions.

If it is assumed that during the maximum rate of gas uptake, the culture was utilizing the gas mixture in the typical proportion of $6H_2$: $2O_2$: CO_2, then the rate of CO_2 uptake was 2.2 l/l culture/hr. To balance the volume of CO_2 respired by one man (22 l/hr), a culture volume of 10 l would be required. The daily output of urine by the subjects used in this study averaged 1.5 l; therefore, this amount of urine diluted 8.9 fold (based upon 11.25 percent as the actual urine concentration) yields a total volume of 13.4 l of culture—theoretically more than sufficient to achieve a balance between CO_2 respired and urine excreted. This same volume of culture would produce a dry-weight cell yield of 90g.

Judging by the low levels of poly-beta-hydroxybutyrate and the high concentration of protein contained in the cells at the termination of growth, growth conditions were favorable with respect to oxygen concentration and availability of nitrogen. Thus the data appear to be applicable to the conditions that occur in continuous culture.

The optical density of the culture continued to increase in a linear manner even after the maximum gas-uptake rate had been reached,

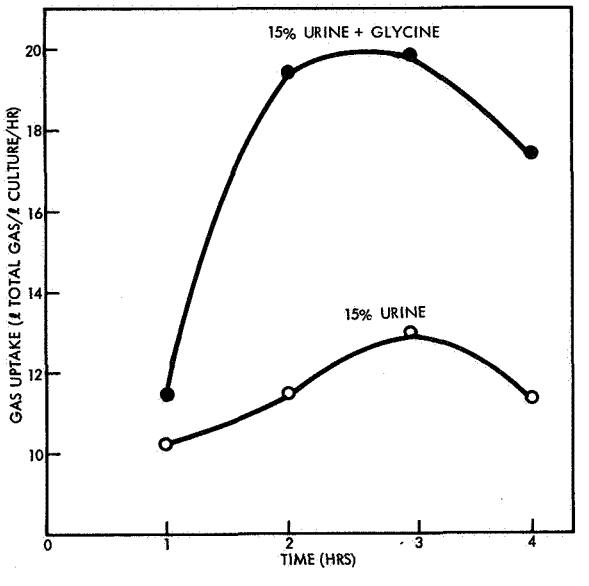

FIGURE 9.—Effect on glycine on gas uptake in 15 percent urine.

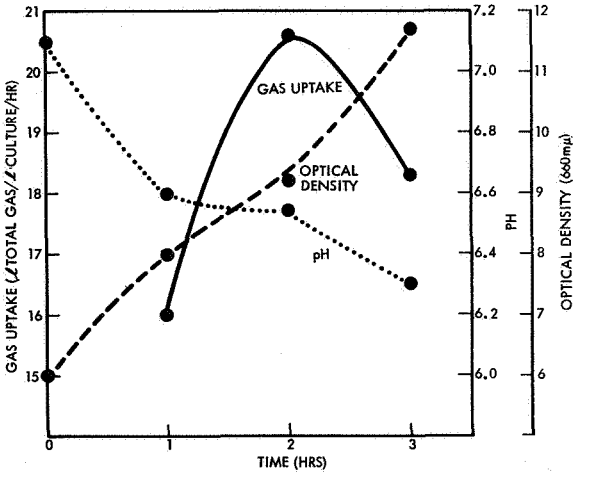

FIGURE 10.—Culture performance in 15 percent urine.

indicating either that storage products were accumulating during the latter portion of the growth period or that the conditions optimum for gas uptake were not identical to those for growth (as measured by optical density).

TABLE VI.—*Culture Characteristics in 15 Percent Urine*

Item	Value
Medium	15% urine + 3X Repaske's trace-elements solutions.
Gas phase	8.3% O_2, 10% CO_2, 81.7% H_2.
Gas-flow rate	22 l/hr.
Impeller stirring speed	2000 rpm.
Growth period	3 hrs.
Maximum rate of gas uptake.	20.6 total gas/l culture/hr.
Initial optical density	6.0.
Final optical density	11.7.
Initial dry weight	2.1 g/l.
Final dry weight	6.7 g/l.
Initial PHB	3.3 (% dry weight).
Final PHB	9.4 (% dry weight).
Final protein (N × 6.25)	70%
Final viable cell count	1.0×10^{10}.

The pH of the culture rapidly declined during the first hour of growth and then stabilized at 6.5 to 6.6. Coinciding with the termination of the maximum rate of gas uptake, the pH again sharply declined. It has been observed that, in general, the pH of the culture appears to be dependent upon the level of urea present. Because *H. eutropha* converts urea to NH_4^+, an equilibrium exists in the culture between H_2CO_3, originating from CO_2 in the gas phase, and NH_4^+. In the early stages of growth, when metabolism and hence urease activity is minimal, the pH of the culture shifts toward the acid side. Toward the latter portion of the growth period, the urea is virtually exhausted, and the pH again drops sharply. Accordingly, in undiluted urine or urine supplemented with urea, the pH remains close to neutrality during most of the growth period. During the latter stages of growth, the pH begins to rise as the efficiency of NH_4^+ utilization diminishes.

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COMMENTS

Dr. REPASKE. Dr. Goldner, is it just a coincidence that, at the pH of about 6.5, your rate of gas utilization goes way up? You start with a pH of about 7.2 and your gas utilization is relatively low.

Dr. GOLDNER. Yes.

Dr. REPASKE. And then as your pH drops, the gas utilization reaches its peak. As you slide off to 6.5 the gas utilization goes down. I was just questioning whether there is any correlation.

Dr. GOLDNER. I am not sure. I do think that at this point urea becomes exhausted and, therefore, carbonic acid begins to affect the pH of the medium more than the balancing effect of ammonia being produced by urease. I think that is probably the coincidence; when you have an adequate amount of urea, or supplement urine with urea, we do not see this drop off. We see a steady state or even a slight decline.

Dr. REPASKE. What happens to the gas uptake curve under those circumstances?

Dr. GOLDNER. It remains on a plateau and extends.

Dr. REPASKE. So again, by coincidence, as the pH stays constant your gas uptake stays constant?

Dr. GOLDNER. Yes, except if we might consider that there is a relationship between simply the pH and the rate of gas uptake. We have done some studies where even with pH as high as 7.5 or as low as 6.0 you can get a plateau of maximum rate of gas uptake for an extended period of time. It seems to be independent of the pH. In other words, the optimum is within a wide range.

Dr. FOSTER. I am worried about your gas rate from the standpoint that you do not agree with mine at all. We have never seen with our densest and most actively growing cultures anything above 9 liters per hour per liter of culture.

Dr. GOLDNER. This is 9 liters of total gas? In continuous culture?

Dr. FOSTER. Yes. Six liters of hydrogen and 2 of oxygen in a batch culture at the peak.

Dr. GOLDNER. Is it in Bongers' medium?

Dr. FOSTER. Yes. I will show it later, but I am worried about some gross discrepancies in the units used.

Dr. GOLDNER. I think the discrepancy may be not so much a function of the medium but, perhaps, the general cultural conditions. For example, the concentration of dissolved oxygen is extremely critical.

Dr. FOSTER. I will call attention to that. But, I wanted to point to your data as being far different from what I am going to show later.

Dr. COOKE. Did you test the effect of mammalian hormones on gas uptake such as steroids as might be found in the urine?

Dr. GOLDNER. No. Inasmuch as most of our work was done on 15 percent urine, you realize that most of the organic fraction is diluted out. Fifteen percent urine does contain an organic fraction but is approximately less than 0.1 percent. It is in the 1/100ths percent. Considering that in undiluted urine these hormones are found in such minute quantities, we were not seriously concerned with them at this phase. What interactions may indeed occur, I do not know.

Dr. COOKE. I noticed on some of the graphs that you approached a steady state. But, you maintained your cultures only for 6 hours or so. Have you run any longer than that? How long can you keep a steady state?

Dr. GOLDNER. Six to 7 hours seems to be the maximum period of time, provided that you supplement 15 percent urine with the nutrients that I have indicated. We have not run any longer. Generally, we take down our culture after the gas uptake declines, because we are interested in analyzing the spent medium.

Dr. BONGERS. Did you ever try to inoculate, or start out with a culture that was previously grown in urine?

Dr. GOLDNER. In all cases we grow the culture, our stock culture, in urine. There is no problem of adaptation. We subculture on a weekly basis. This has been going on for over a year.

Dr. SCHWARTZ. Have you any evidence that you are getting magnesium ammonium phosphate precipitating and coming down in to the cells, as a way of accounting for part of this balance?

Dr. GOLDNER. Yes. That certainly is a problem. I have not noticed that it is obvious to sight. There may be some doubt, however, in the cells that you cannot see. That may be a consideration. It may account for the apparently depleted amount of phosphorus. However, I would expect the condition to occur under all circumstance, not just certain circumstances. It does not seem to be a constant effect.

Dr. REPASKE. Have you been able to substantiate the observation about nickel being a requirement?

Dr. GOLDNER. We have not looked at it. Apparently, I thought Bongers had more or less settled the question, that in the presence of ferrous iron, nickel is no longer required. I did not go further in our study.

Dr. REPASKE. The only other point I would like to make is that your observations with contaminants are the same that I have observed.

Dr. GOLDNER. Have you ever seen a case of stimulation as a result of contaminants?

Dr. REPASKE. No. Usually if I assume a culture is pure, and my stock inoculum has become contaminated, I can tell immediately because the culture does not respond the way it should. A microscopic examination confirms it.

Dr. GOLDNER. It is often very difficult to observe contamination. We routinely plate out our cultures on Trypticase Soy agar, which is excellent. But many times it takes several days before the contaminants show up even under those conditions.

Dr. REPASKE. Does it usually show up as a satellite?

Dr. GOLDNER. It shows up as a satellite or it is present right in the streak. Satellite conditions are pretty rare. Usually it shows up in the streak itself.

Dr. CALLOWAY. I want to comment on the question of trace minerals. The diets that we use are supplemented with chromium, selenium, zinc and manganese and others, as pure salts. If you ever have a chance to get all the trace minerals going in urine, it is probably in these samples. We probably have nickel in there, because one of the constitutents of the diet is hydrogenated lipid. They almost always use a nickel catalyst to make it. We have not looked for any of these things because, of course, a lot of them will be in the stool instead of the urine. Nevertheless, they are in the diet at least.

The one thing that is not in the diet that you may have to cope with is the endogenous amount of uric acid. We have absolutely no nucleic acid.

Dr. TISCHER. On that point I would like to comment that we have available pyrogen-free water with no more than 40 parts per billion of salts of any kind in it. If this is of any use to you, we can furnish it in small quantities, unless you have some of your own.

Dr. GOLDNER. I think we are going to have to develop a technique for such a capability. Considering what happens with commercial grade distilled water, we would be well advised to consider the quality of water very carefully.

Dr. TISCHER. You would be well advised to consider the cost of it, too, because water of 15 to 18 megohms resistance is not cheap.

Dr. DECICCO. Dr. Goldner, I noticed that your lipid on that one composition chart was very low—3.3 percent at the beginning, and then about 9 percent after-

wards. This then is presumably your inoculum shortly after inoculation when it is so low.

Dr. GOLDNER. Yes.

Dr. DECICCO. Does the cell use that inoculum? What stage are these in? I would have expected they would be high in lipids.

Dr. GOLDNER. I would too. They are grown in closed flasks. Of course a vacuum develops, so it is not optimum for growth. Apparently we are catching them very early in the stationary phase.

According to the method of Williams and Orgenson for PHBA, digesting the cells with hypochlorite, these are the analyses that we show. They seem to be very reproducible, within the limits of the analysis. There may be other factors present, or other storage products. But this is what our PHBA shows.

Current Problems in the Cultivation of *Hydrogenomonas* Bacteria Under Controlled Environments in the Turbidostat

JOHN F. FOSTER AND JOHN H. LITCHFIELD
Battelle Memorial Institute
Columbus, Ohio

This paper describes recent experiments on the continuous culture of *Hydrogenomonas eutropha* in our laboratory's turbidostat. In our experimental culture system, a constant culture environment is automatically maintained within preselected limits of the controllable variables. These variables include cell density, pH, temperature, CO_2, H_2, O_2, and urea concentrations. The added materials and the cellular products are measured quantitatively to permit an estimate of material balances and conversion efficiencies. Interpretations of these results are discussed in terms of extracellular interactions among environmental components and in terms of growth mechanisms consistent with the response of the organism to its chemical and physical environment.

The preceding reports of this series (refs. 1 and 2) describe the continuous culture equipment, research study objectives, and operating characteristics of the Battelle Auto-Culture System. The effects of environmental variables on assimilation of gases and urea, growth rate, and cell composition are discussed in qualitative terms. A range of optimum values was estimated for each variable, insofar as such optima could be specified at that time.

In the 7 months since our report at the last conference, 18 experimental runs have been made in the Auto-Culture System. Each run extended for 3 to 5 days, except for those few that were interrupted prematurely by operational faults. Procedures and measurements have been improved continuously as soon as the need became apparent and the methods available. With more precise and varied data available for interpretation, our concept of a growth system embodying complex mechanisms and interactions has grown, our capability for anticipating system requirements has improved, and our progress toward the primary objective of maintaining a steady rapid growth in dense cultures has been encouraging; however, the ultimate goal has not yet been reached.

EXPERIMENTAL RESULTS

Treatment of Data

Each experimental run generates a massive amount of raw data on environmental conditions and material input rates. These are measured and recorded, either continuously or once each minute. Reduction of these data to an interpretable form must be done manually, because judgment must be used in the application of instrument constants to the charted records to convert the data to gravimetric units for material balances. The instrument and sensor constants are based on periodic calibrations with known standards, but constants sometimes drift between calibrations. Usually an accurate interpolation can be applied by examining concurrent measurements of other variables. This examination detects and makes allowance for real changes in the culture system. Then trial material balances can usually estimate the true value of the constants between direct calibrations, and a valid graph can be prepared showing all culture characteristics as real-time functions. Although programs for data reduction are gradually being developed, they are not yet suitable for computer use.

Individual summaries of data from 15 or more weeklong experiments are too extensive to be presented in this paper. As a preferred alternative, the experimental data from the most recently analyzed run (23035–75) are described in detail and discussed with reference to the ac-

cumulated experimental experience of earlier runs.

Experimental Plan

In all the experimental runs, the overall objective was to reach a high cell density and maintain active growth, so that a steady state could be established with high substrate-conversion efficiencies. A period of batch growth without dilution was inaugurated for varying periods following inoculation to increase cell density as rapidly as possible. Then dilution was started at a rate less than the growth rate, and the cell density continued to rise as long as growth continued at the initial rate. Inevitably, growth rate decreased as cell density increased, presumably because the culture environment was not adequately maintained in an optimum state to supply culture requirements.

Characteristic Interactions in the Culture

Interactions among the culture and the environmental components produce responses that have come to be recognized in the course of the experiments as characteristic and qualitatively reproducible. These are summarized here to introduce the detailed discussion that follows:

1. The pH of the culture usually passes through a characteristic cycle during initial growth phases. The pH cycle starts with a broad maximum, a sharper minimum, and then a rapid increase that continues and must be controlled by adding acid.
2. The most rapid growth occurs before the pH spontaneously starts to decrease following its first maximum.
3. Gas absorption by the culture increases in proportion to the increasing cell density until the pH rises the second time. Then there is a sharp decrease in gas input rates.
4. The growth rate decreases after the pH passes through its minimum value.
5. Urea conversion increases abruptly as the pH passes through the sharp minimum.

Two Batch Cultures in Run 23035–75

Earlier runs had shown that the turbidostat worked perfectly in maintaining a constant culture cell population based on a measurement of the optical density of a sample diluted in known proportions. Whenever the measured optical density at 650 mμ exceeded the control point, the apparatus added enough fresh nutrient to dilute the culture to the control point. Simultaneously, an equal amount of culture was removed to maintain a constant volume. This run was set up with the immediate objective of studying batch growth after inoculation to learn how a dense culture in an active growth state might be produced in the shortest possible time. Figures 1 and 2 summarize the experimental results from two consecutive batch cultures that gave similar results. Each was inoculated, without opening the culture vessel, by draining the system after the preceding experiment, refilling with fresh nutrient, and allowing traces of the culture to remain as inoculum in the connecting tubing of the sampling system. Thus Batch 1 of this experiment was inoculated from the preceding Run 23035–68, in which the organism was adapted to similar conditions, and Batch 2 was inoculated similarly from Batch 1 of this experiment. The temperature was maintained at 30°C; the gas phase contained O_2, CO_2, and H_2. Initially, the urea concentration was 0.75 gram per liter. The nutrient medium was lightly buffered at pH 6.5 with KH_2PO_4 at 0.136 gram per liter and K_2HPO_4 at 0.05 gram per liter.

Each figure gives a semilog plot of five variables: pH, cell density, gas input rates, urea concentration, and CO_2 partial pressure. Clock time in hours is shown as a linear scale on the abscissa. In order to accommodate all the system variables on the same numerical log scale, both cell density in grams (dry weight) per liter and urea concentration in grams per liter are divided by a factor of 10. The gas input rates are given in liters at standard temperature and pressure per hour per liter of culture, with the individual input rates for H_2, O_2, and CO_2 divided by factors of 6, 2, and 1, respectively, because the three gases are always converted in approximately these ratios. Thus the gas-input-rate curves would all fall on the same curve if the ratios were exact, and significant variations from these ratios are easily apparent. A plot of CO_2 partial pressure in atmospheres is included because the CO_2 concentration in the gas phase varied during a part of the run when CO_2 was

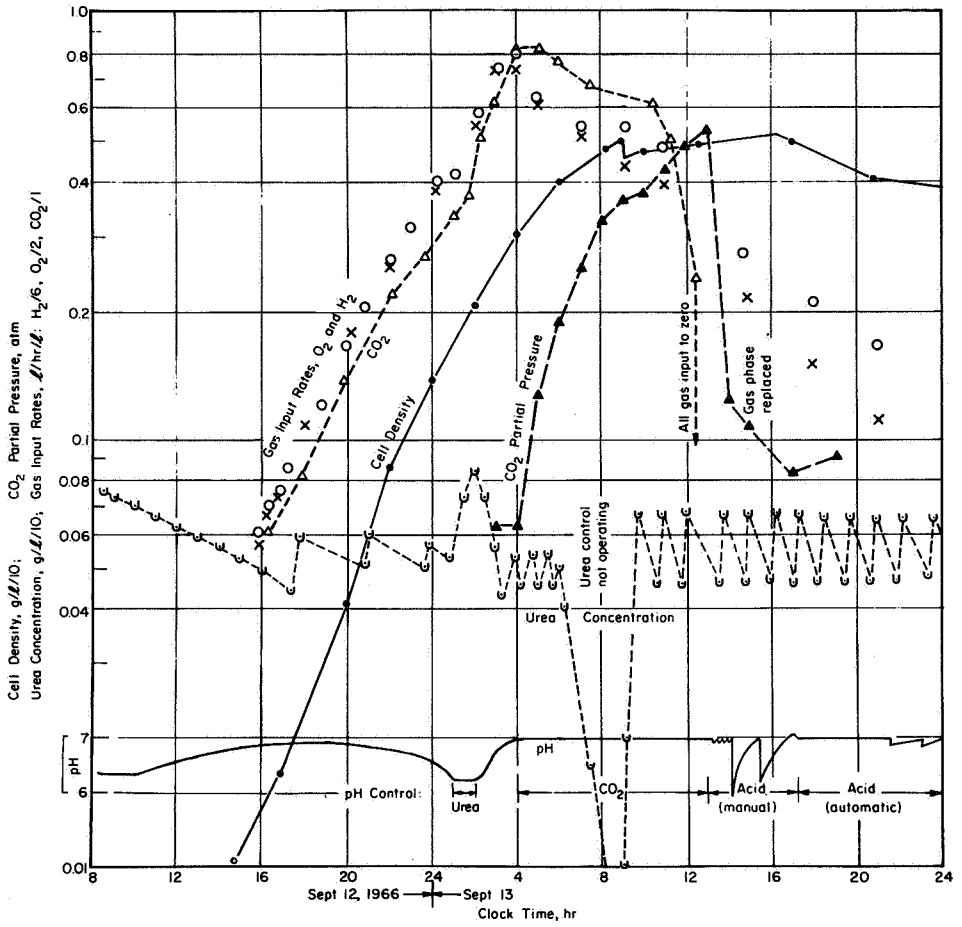

FIGURE 1.—First batch culture of H. eutropha, run no. 23035–75.

used to prevent the pH of the culture from increasing above the maximum of 7.0.

Culture pH

The initial measurement of the first batch culture pH (fig. 1) was 6.3 at 9/12–1000, which reflects the influence of the dissolved CO_2 in the nutrient medium that was originally at pH 6.5. The pH curve is extrapolated backward to 0830 at the same value to indicate that inoculation took place at this point, although the pH was not actually measured. The pH rose slowly for 8 hours until it reached 6.9 at 1800. Then it reversed and fell to 6.2 at 9/13–0100. At this time the automatic equipment added urea at a rate sufficient to compensate for falling pH, and a minimum of 6.2 was maintained for 1 hour. It should be noted that urea is neutral in reaction and that the pH was undoubtedly limited at the minimum value by a decomposition product from urea (presumably ammonia). Then the pH trend spontaneously reversed and rose within 2 hours to the maximum of 7.0 where the pH was limited automatically by the addition of CO_2 on pH demand between 0400 and 1300. At this point the CO_2 concentration had risen so high that the system was manually switched from CO_2 to acid control of pH. Acid control was maintained for the rest of the experiment. The amount of acid required was not measured quantitatively, although it was considerable. For example, between 1700 and 2130, 470 ml of 0.1 NHCl were required.

Figure 2 shows a similar pattern of pH variations. The pH recorder was activated at the time of inoculation and indicated a pH of 5.8 after the nutrient was saturated with CO_2 from the gas phase containing 6 percent CO_2. The

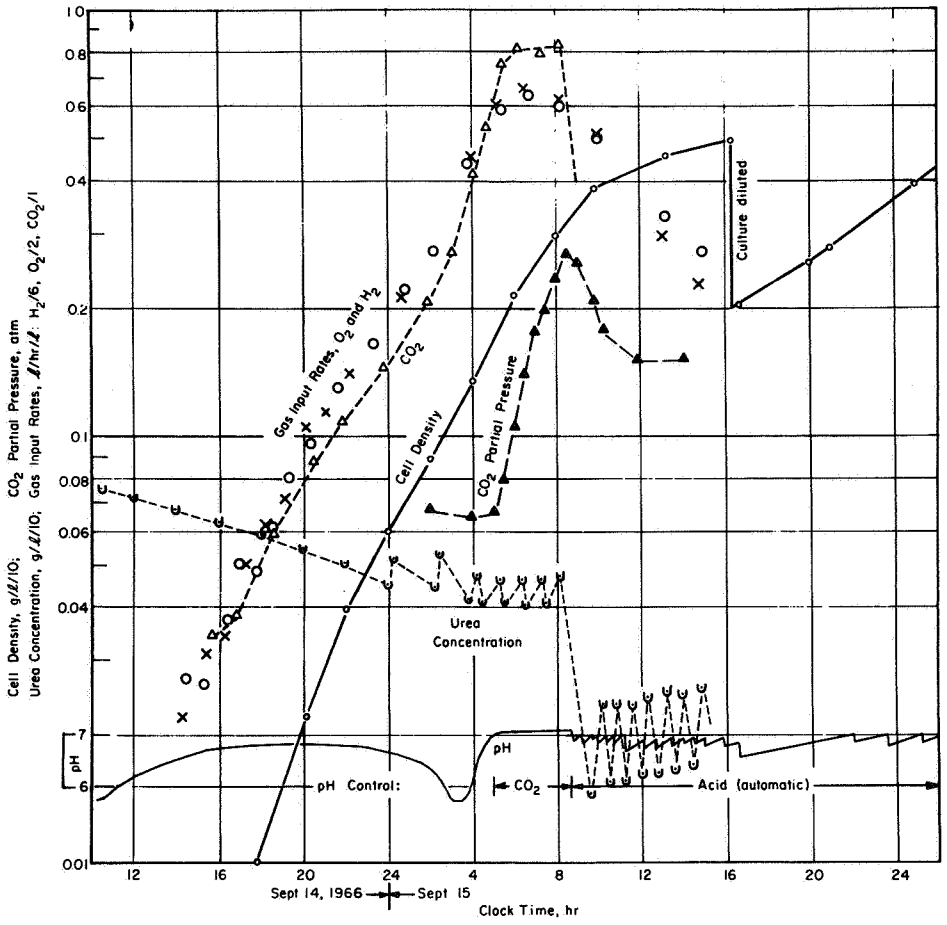

FIGURE 2.—Second batch culture of H. eutropha, run no. 23035–75.

initial rise and fall of pH covered an identical time period of 17 hours to a minimum of 5.8 at 9/15–0300. In this batch run the minimum pH was not limited by urea additions because the urea pH control was not activated. The minimum was anticipated at a time in the early morning when the apparatus was unattended, and it was believed undesirable to depend on automatic control completely because urea additions do not always control minimum pH. Once again the pH rose rapidly in about 2 hours to a maximum of 7.1 where the automatic limit control added CO_2 until it was manually switched over to acid control. In this case it was believed undesirable to allow the CO_2 concentration to rise as high as had been the case in the first batch run. Frequent acid additions were made automatically for the rest of the run, using 1 N HCl to minimize the amount of dilution that occurred.

Cell Density

The cell density is recorded as a continuous curve on the recorder chart. The points shown on the cell density curve of figure 1 represent readings taken from the chart at 2-hour intervals throughout Batch 1 growth, rather than intermittent determinations. Grab samples were measured on another spectrophotometer to check the continuous determination of cell density. Any variations in the optical-density cell constant of the continuous recorder were corrected by these external measurments. Some of the culture sample that flows continuously through the optical-density cell does tend to deposit on the windows and cause a slight drift

of zero as the run continues, but this has not been found to be serious.

The first three segments of the cell-density curve on figure 1 are the periods of most rapid growth between 9/12–1430 and 2200. Then there appears to be a break in the curve with slower growth for two or three more segments, followed by a further decrease in the following segments. The correlation between the most rapid growth period and the pH of the culture is not always clear. Experience with the two batch cultures of figures 1 and 2, and with others as well, indicates that the growth rate decreases after the pH passes through the broad maximum and starts to decrease. We postulate that the decrease in growth rate follows the time of the pH maximum by about one generation time of the organism. A further decrease in growth rate correlates exactly with the sharp break in the gas input rates, and approximately with the time in which the pH reaches the controlled maximum at 7.0 following the sharp pH minimum.

Figure 1 shows that the cell density continues to rise to about 5 grams per liter at 9/13–0900, when a small amount of fresh nutrient was added to bring the culture volume back up to 2 liters, since the volume had decreased slightly because of continous sampling for analysis. Following this addition the cell density continued to rise very slowly until automatic acid addition was started to compensate for the pH rise. The addition of a considerable amount of acid tended to dilute the culture and the cell density decreased for this reason. In Batch 2 (fig. 2) the cell density also increased to 5 grams per liter. Then half of the culture was discarded and replaced by fresh nutrient, which is indicated by the abrupt decrease in cell density to 2 grams per liter. This procedure accelerated the growth rate somewhat, and the culture density increased again to over 4 grams per liter before the experiment was discontinued.

Urea Concentration

At the time of inoculation, the urea concentration was 0.75 gram per liter, and the concentration control was set to maintain a minimum of 0.5 gram per liter. During the initial growth period, the urea concentration fell steadily (fig. 1) for a period of 9 hours until the minimum concentration was reached. Then the automatic controls added urea intermittently to maintain the average concentration at about 0.5 gram per liter for an additional 8 hours. At this time the pH controls took over, and urea was added to maintain pH at the minimum value of 6.2. Urea concentration rose in the next hour to about 0.85 gram per liter, before the pH started to rise spontaneously and the addition of urea for minimum pH control ceased. Urea concentration fell relatively rapidly until the concentration control took over again and maintained the concentration at about 0.5 gram per liter until 9/13–0600. Because urea was being added more rapidly than anticipated, the urea supply was exhausted while the apparatus was unattended, and the urea concentration decreased until no urea could be detected in the culture. When the urea reservoir was replenished, additions were resumed by the automatic equipment and the concentration rose rapidly to the control point. A concentration of about 0.5 gram per liter was maintained in the system for the remainder of the experiment.

The initial decrease in urea after inoculation was at a rate much greater than the corresponding increase in cell density. This means that urea was being converted in the system to some intermediate product, since it could not have been assimilated directly by the organism to the extent that it was disappearing from the culture. The slopes of the segments of the concentration curve in which concentration is decreasing indicate qualitatively the rate of conversion of urea because no urea was being added to the system during these periods. These slopes are relatively constant in value and indicate a lower rate of conversion during the first four segments, before the time that the pH passed through its minimum. Thereafter the slopes are much more steeply downward, which is interpreted as a change in the mechanism of the urea conversion after the pH minimum.

Figure 2 shows a similar pattern of urea-concentration variation with time. There was again a marked increase in the rate of urea conversion at the time of the pH minimum. This changeover in conversion rate at the pH minimum is

much more abrupt than can be shown clearly in figure 2. Examination of the recorder chart shows that the change occurred within less than 15 minutes. Figure 2 shows that the urea-concentration control was reset in Batch 2 at 9/15–0800 to decrease urea concentration in the culture to about 0.2 gram per liter. This was done as an attempt to reactivate the slowly growing culture, but there was no detectable effect on culture growth during the remainder of the experiment.

Gas Input Rates

Gas input rates are plotted in figures 1 and 2 for each of the three gases, using the symbols o for oxygen, x for hydrogen, and $\triangle$ for CO_2. The CO_2 points are connected with a dashed curve to show the trend in CO_2 rates, but curves for O_2 and H_2 are omitted for clarity and only the data points for these two gases are shown. Each plotted point represents the average rate for a measured time period varying between about 30 and 60 minutes, during which a recorded number of increments of the gas were added by the automatic apparatus. Each increment contained a known and constant volume of the required gas. The partial pressures of the three gases were controlled at about 6 to 8 percent for CO_2 and 10 to 12 percent for O_2; the remainder was H_2. Total pressure was 2.5 to 3.5 psig.

The gas input rates increased generally in proportion to the increasing cell density in each of the batch cultures while the pH was passing through its first maximum and the following minimum. When the pH rose the second time to the imposed limit of 7.0, the characteristic sharp decrease in gas rates took place. This break anticipated the decrease in growth rate that occurred 3 hours later. There is a significant variation in trends of the CO_2 input rate that reflects the change in the carbonate-bicarbonate-carbonic acid-CO_2 equilibrium as the pH varies. While the pH is falling, there is progressively less bicarbonate in equilibrium with dissolved CO_2, and the slope of the CO_2 input curve decreases because bicarbonate decomposes to supply part of the CO_2 demand. Thereafter, as the pH rises again, more bicarbonate forms and CO_2 input increases to supply both the increasing bicarbonate and the CO_2 demand of the organism. Finally, CO_2 is being supplied to control pH, and the CO_2 inventory builds up as shown on the CO_2 partial pressure curve. The CO_2 input rate remains relatively high, compared to O_2 and H_2, but CO_2 assimilation by the culture undoubtedly decreases in parallel with the decreasing O_2 and H_2 demands.

Figure 1 shows that all gas inputs decreased to zero about 3 hours after the urea was exhausted in the culture by failure of the urea supply. However, the CO_2 partial pressure continued to increase to a maximum of over 50 percent, in order to control pH. In an attempt to establish growth again in the culture, the gas phase with excess CO_2 was replaced by flushing with H_2 and then reactivating the gas controls to maintain about 10 percent CO_2 in the gas phase. Oxygen and hydrogen demands subsequently were reestablished at a lower rate which continued to fall because the culture evidently was not growing. No further CO_2 demand could be detected and the experiment was terminated.

Figure 2 shows that Batch 2 passed through much the same sequence, but the urea concentration was maintained at about 0.5 gram per liter and the CO_2 partial pressure increased only to about 28 percent before the urea control was reset to maintain about 0.2 gram per liter in the culture. Thereafter, the O_2 and H_2 demand continued to fall and the CO_2 demand was so low that it was supplied from the CO_2 inventory and no further CO_2 input was recorded. Growth continued slowly until the culture was diluted. Then the growth rate increased somewhat but never attained the original high rate. Gas inputs were also reestablished in response to the demands of the growing cultures following dilution, but these gas data have not been plotted in figure 2.

The O_2 input shows a significant temporary increase in O_2 rate concurrent with the pH minimum in both figures 1 and 2. This is apparently correlated with the simultaneous change in the rate of urea conversion caused by some shift in the mechanism of conversion of the input materials. No detailed explanation of these characteristic observations is yet available, although there may be an enzymatic feedback that increases conversion of urea to ammonia rather than to some other intermediate product.

Material Balance

Examples of a hypothetical buildup and depletion of inventories of N_2 and C_2 compounds in the two batch cultures are illustrated in figure 3. This figure shows plots of calculated differences between the measured inputs of C_2 and N_2, as urea and CO_2, and the C_2 and N_2 contents of the cellular products, during identical time periods. In each culture, the only identifiable nitrogenous product was the incremental weight increase in the number of cells. The only N_2 input was the N_2 content of the urea. This was calculated from the difference in urea concentration at the beginning and end of each time interval plus the urea added during the interval. Figure 3 shows the cumulative difference between N_2 input and N_2 in the cell product, assuming that the cells contained 12.2 percent nitrogen. This percentage is the approximate average of N_2 in actively growing cultures, based on analyses of cells from other experiments. Similarly, the cumulative carbon inventory is the difference between carbon in CO_2 and in urea supplied to the culture during the selected time period, and the cellular carbon, which was assumed to be 53 percent of the dry weight of the cells produced. Also plotted in figure 3 are the pH curves representing the variation in pH with time that are identical with the pH curves shown in figures 1 and 2. The value of the growth-rate constant also varies with time, as noted before, and is plotted at the appropriate scaler level in figure 3.

As a result of these calculations, both the hypothetical N_2 and C_2 inventories increase concurrently with pH. In Batch 1, the inventories rise more rapidly than in Batch 2 and pass through a maximum at approximately the same time as the pH maximum. The lack of exact congruence between the maxima may not be real but may reflect an error in the assumptions as to the carbon and nitrogen composition of the cellular product. In Batch 1, also, the inventory tends to fall as the pH falls towards its minimum. Then there is an abrupt minimum in both the N_2 and C_2 inventories at about the same time as the pH minimum. In Batch 2 the inventory rises more slowly toward a maximum that is broadened and not fully developed in the N_2 inventory and is never completely evident in the C_2 inventory. In both batches the growth rate rises and falls again in approximate corre-

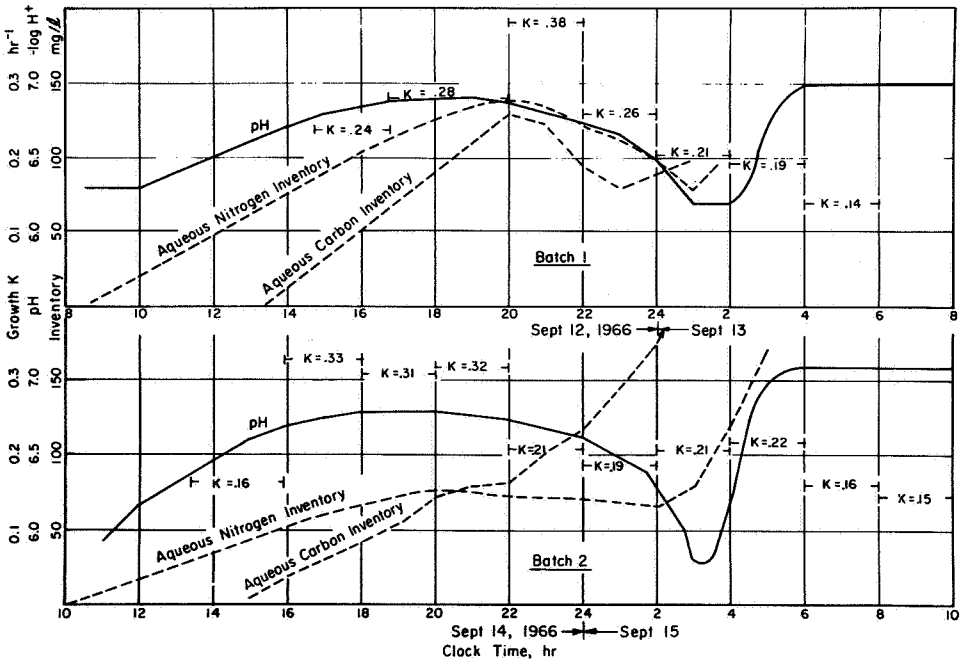

FIGURE 3.—Material balances for batch cultures of H. eutropha, run no. 23035–75.

spondence with the N_2 inventory. These relationships appear qualitatively significant although they may not be quantitatively reproducible.

DISCUSSION

The experimental results presented here are characteristic of many other experiments in which the growth of the culture spontaneously decreased after a similar pH cycle. The calculations illustrated in figure 3 indicate that urea is transformed into an intermediate product before its ultimate conversion into cellular material. It is hypothesized that this product is beneficial to growth, since the growth rate peaks at about the same time. It is possible that, in the later stages of culture, a feedback control mechanism induced in the organism by its environment causes much more rapid conversion of urea. This rapid conversion has the characteristics of a decomposition that produces ammonia as a direct product from urea. It cannot yet be determined whether or not the initial conversion also forms ammonia at a lower and more easily assimilated concentration, or forms another intermediate that stimulates growth. In either event, under the environmental conditions described, the organism exhibits maximum growth rates higher than have been observed before. Therefore, these results provide significant guidance for the study of optimum growth conditions in future continuous culture experiments.

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COMMENTS

Dr. JENKINS. How is your 20-liter unit coming along?

Dr. FOSTER. It was recessed for a while. We are continuing construction on it now.

Dr. JENKINS. Do you have any idea when it will be in operation?

Dr. FOSTER. We need twice as many men to run the 20-liter, and we are not sure just how it ought to be run or what controls ought to be put on it. I think that this is more critical. We have made the break now, but it takes a great deal more labor to keep a 20-liter supplied than a 2-liter.

Dr. KRAUSS. Can you tell us how you are monitoring your ammonia and urea continuously?

Dr. FOSTER. It is a Standard Technicon clinical analysis for blood urea and nitrogen. The ammonia analysis is not standard.

Dr. MILLER. On urea analysis do you remove the cells before it goes into the analyzer?

Dr. FOSTER. No. It is dialyzed across the membrane by the standard blood; they do not take out the blood cells either. We found no effect.

Dr. REPASKE. Do you have any idea whether the urease is extracellular or whether it is part of the cells?

Dr. FOSTER. I would say it must be extracellular because this urea decomposition continues indefinitely after the cells still seem to be growing. They may be taking up hydrogen and oxygen, but they are in a resting phase.

I do not know with certainty whether it is extracellular or not. We have done some analyses of the substrate for urease. They show some, but I do not regard them as quantitative.

Dr. REPASKE. It would be interesting to take some spent culture medium and throw in some urea and see if you get any carbon dioxide.

Analysis of Continuous Microbial Propagation

A. G. Fredrickson and Henry M. Tsuchiya
University of Minnesota
Minneapolis, Minnesota

MATHEMATICAL MODELS FOR GROWTH OF MICROORGANISMS

The behavior of a population of cells is connected with the behavior of the individual cells. If we know the connection, we can learn about cell behavior from cell-population behavior. Conversely, cell behavior can predict cell-population behavior. The behavior of cells and cell populations and, therefore, predictions of same are expressed in quantitative terms. Hence, mathematical models are needed. In this instance, the mathematical model is simply a set of hypotheses—translated into mathematical terms—about the processes going on in single cells, and how these processes interact with and change the cellular environment. The hypotheses of the model are formulated by plausible inference from existing biological and physical data and principles.

The mathematical model must be tested by experiment; this involves:

1. Finding numerical values of constants and parameters by performing an appropriate set of experiments.
2. Studying the model to deduce its quantitative predictions about different experimental situations.
3. Comparing prediction and observation.
4. Revising or rejecting the model if prediction and observation do not agree.

This paper reports work done on the formulation of general models for continuous microbial propagation.

THE NON-SEGREGATED MODEL OF THE CONTINUOUS PROPAGATOR

We shall be concerned with the dynamics of continuous propagation of a microbial culture. Figure 1 suggests the general model for this process. The material in the propagation vessel is divided into three phases: the gas phase, the liquid phase, and the biological "phase." (The last is the material contained within the boundaries of living cells, and so is obviously not a phase in the usual sense of the word.) We treat each phase as a continuous collection of matter. That is, we disregard the segregation of the gas phase into bubbles dispersed throughout the liquid phase, and disregard the segregation of the biological phase into discrete units (cells) dispersed throughout the liquid phase. We call the model "non-segregated." Obviously, these idealizations introduce some inexactitude.

Figure 1 suggests that control may be exerted on the input streams to the propagation vessel. Depending on the kind of control used, continuous propagators have been called "chemostats," "turbidostats," "bactogens," etc. In the chemostat, the flow of fresh nutrient to the vessel is held constant, irrespective of the state of affairs within the vessel. Thus, there is no external feedback control. In the turbidostat, the flow of fresh nutrient to the vessel is controlled by the turbidity of the effluent stream (the external feedback control). Some control may also be exerted on the flow of gases to the vessel. Apparently no classification of propagators according to the kind of gas-flow control has been proposed. Mathematical equations may be deduced for the nonsegregated model by application of well-known physical and biological principles to the model. These principles are:

1. The principle of conservation of mass of chemical species, applied to all three phases.
2. The principles of stoichiometry applied to the biochemical reactions in the biological phase.

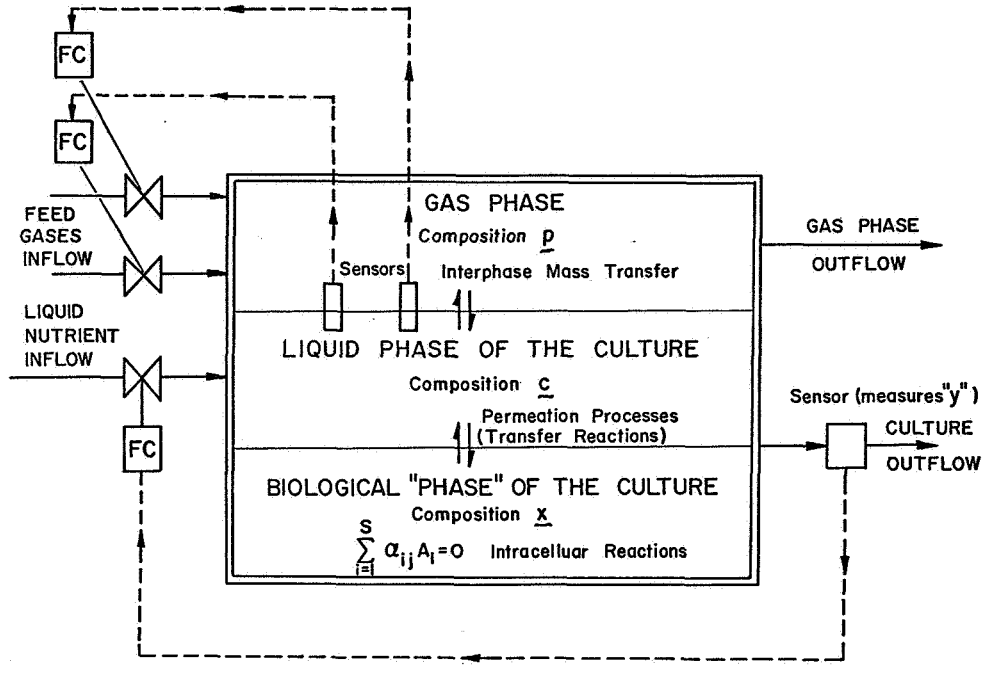

FIGURE 1.—Schematic diagram of a continuous microbial propagator.

3. The principles governing interfacial mass transfer.

The equations that result can help us understand the dynamics of continuous propagation. To develop these equations, we must first find the necessary parameters.

MEASURED AND NON-MEASURED VARIABLES; "HIDDEN" VARIABLES

It is almost unnecessary to say that a model should not contain unmeasureable quantities, but it may well contain quantities that are not measured. This is true of the nonsegregated model; the said quantities may profoundly influence the dynamics of continuous propagation.

Consider the biological phase of the continuous propagator. This will consist of a large number of different kinds of molecule, ranging from small ones (such as sugars and amino acids) to very large ones (such as DNA and protein). The concentrations of these various molecular species in the culture (liquid phase plus biological phase) are measureable, and denoted by $x_1, x_2, \ldots, x_I$. That is, x_i represents the amount (say in moles) of the i^{th} molecular constituent of the biological phase in unit volume of the culture. Subsequent manipulations are facilitated by matrix notation:

$$\mathbf{x} = \begin{bmatrix} x_1 \\ x_2 \\ \cdot \\ \cdot \\ \cdot \\ x_I \end{bmatrix},$$

where $\mathbf{x}$ is called the *concentration-of-biochemicals vector*.

Each of the elements x_i of the vector $\mathbf{x}$ is measurable, but it is practically impossible to measure all of the x_i in any given experimental situation. In many investigations, only a single quantity is determined as a measure of culture density. The methods commonly used for this purpose (such as dry weight measurements or turbidity measurements) are tantamount to measuring a linear combination of the x_i. Thus, if y is the generalized culture density (that is, the measure of the culture density employed in a particular experiment) it can be written as

$$y = \mathbf{m} \cdot \mathbf{x} \tag{1}$$

where $\mathbf{m}$ is a constant-row vector whose elements are the weight factors for the x_i in the definition of y. For instance, if y happens to

be the dry weight of biomaterial per unit volume of culture, then m_1, m_2, . . ., m_{I-1} are the molecular weights of chemical species A_1, A_2, . . ., A_{I-1}; but if A_I is water, then m_I is zero. The necessity for introducing the generalized culture density arises because in turbidostat-type operation, y is sensed and used to control the flow rate to the propagator.

The generalized culture density is, of course, not the same as the population density—the number of cells per unit volume of culture. In the non-segregated model, nothing is said about the population density.

Consider next the liquid phase of the culture. Let c_1, c_2, . . . , c_L denote the concentrations, expressed as moles per unit volume of culture* (liquid phase plus biological phase) of the various chemical species in the liquid phase Thus, the various c_i will determine, among other things, the pH of the culture, the concentration of salts and products of metabolism, and the level of dissolved gases in the liquid. Again, it is convenient to define

$$\mathbf{c}=\begin{bmatrix} c_1 \\ c_2 \\ \cdot \\ \cdot \\ \cdot \\ c_L \end{bmatrix}$$

and call **c** the *concentration-of-(liquid) environment vector.*

Finally, the composition of the gas phase is specified by a vector of partial pressures, **p,** which it is unnecessary to define further here.

The parameters contained in the three vectors **x, c,** and **p** represent measurable quantities. Application of the three basic principles noted earlier will allow us to arrive at a set of equations relating these three vectors to each other. The equations are, of course, the dynamical equations of the propagator, and describe the development of the vectors **x, c,** and **p** in time, as well as their response to external stimuli.

To make the dynamical equations really useful, we must assume that they are complete. That is to say, no variables other than those in **x, c,** and **p** appear in them. An example of a non-segregated model based on the foregoing assumption is that of Monod (ref. 1); more sophisticated models of the same type have appeared in the literature (refs. 2, 3, and 4).

The assumption that the dynamical equations for **x, c,** and **p** are complete is questionable. Every population is a distribution of individuals of differing states. Assuming that the dynamical equations for **x, c,** and **p** are complete is really assuming that the distribution of individuals is completely characterized by its mean, and that higher moments of the distribution do not affect the dynamic behavior of the population. In general, of course, these higher moments will affect the dynamic behavior of the population. They therefore represent hidden variables; that is, variables that should be measured and included in dynamical theories but usually are not.

The point of the foregoing considerations may be illustrated by analogy with a simple example. Suppose we wanted to know the settling rate of a suspension of solid particles in water, and that the distribution of particle sizes followed a Gaussian distribution. Two parameters—the mean and the variance—are needed to completely specify the Gaussian distribution. If we knew just one parameter, the mean, we might or might not be able to make a reasonable estimate of the settling rate. If we knew both the mean and the variance, then (in principle) we could accurately estimate the settling rate. The variance would be the hidden variable.

We have developed the concept of hidden variables at greater length elsewhere (ref. 5). Here, we shall ignore the effect of hidden variables and so assume that the dynamical equations for **x, c,** and **p** are complete. At present, we do not know what inaccuracies this assumption can lead to.

INTERFACIAL MASS TRANSFER

In chemical engineering, it is customary to consider the rate of transfer of chemical species from a gas phase to a liquid phase, or vice versa, as the product of two factors. The first factor is a so-called "overall transfer coefficient" (and is itself the product of two factors, one of which

*If c_i is the concentration expressed as moles per unit volume of liquid, then $c_i=c_i/(1-\phi)$, where ϕ is the volume fraction of biological phase in the culture.

is the interfacial area per unit volume of culture, and the other is a conductance). The second factor in the transfer rate is a driving force, and is the difference between the actual concentration in the liquid phase, and the concentration that would exist if the liquid phase were in equilibrium with the gas phase. Thus, the rate of transfer of the i^{th} chemical species per unit volume of culture is written.

$$\frac{1}{1-\phi}\sum_{l=1}^{L} K_{il}(c_l^*-c_l),$$

where the K_{il} are the overall transfer coefficients, L is the number of chemical species of interest, and c_l^* is the concentration of the l^{th} species in a liquid in equilibrium with the gas phase. The overall transfer coefficients K_{il} depend on a complex of factors, including level and kind of agitation, viscosity of the culture, and the diffusion coefficients of the various chemical species in the liquid phase. They are discussed at greater length by Finn (ref. 6), Aiba, Humphrey, and Millis (ref. 7), and Toor (ref. 8).

INTRACELLULAR CHEMICAL REACTIONS AND PERMEATION PROCESSES

Let A_i represent the i^{th} kind of molecular species composing the biological and liquid phases of a culture. A_i is not a quantity; it is simply a shorthand way of writing DNA, RNA, $C_6H_{12}O_6$, etc. Suppose that $A_1, A_2, \ldots, A_I$ represent the chemical substances composing the biological phase, whereas $A_{I+1}, A_{I+2}, \ldots, A_S$ represent the chemical substances composing the liquid phase. If the same substance appears in both phases, it is convenient to give it a different symbol for the two phases. For example, A_1 might represent water (free) in the cells; A_{I+1} might represent water in the liquid phase of the culture. The stoichiometry of a set of R reactions is then neatly summarized by a set of "reaction equations"

$$\sum_{i=1}^{s} \alpha_{ij}A_i=0, \ (j=1, 2, \ldots, R) \qquad (2)$$

where the α_{ij} are stoichiometric coefficients such that

$\alpha_{ij}>0$ if A_i is produced by reaction j,

$\alpha_{ij}=0$ if A_i does not participate in reaction j,

$\alpha_{ij}<0$ if A_i is consumed by reaction j.

The foregoing notation is described more completely by Aris (ref. 9).

In some of the reactions described above, chemical substances of the liquid phase (A_{I+1}, $A_{I+2}, \ldots, A_S$) will not appear, because their stoichiometric coefficients are zero. We call these intracellular reactions. All other reactions, involving chemical species from both the biological and liquid phases, are called transfer reactions, for obvious reasons.

Let r_j ($j=1, 2, \ldots, R$) be the rate of the j^{th} reaction expressed as moles per unit time per unit volume of culture. The r_j must be assumed to depend on all elements of the vectors $\mathbf{x}$ and $\mathbf{c}$. (In addition, they may depend on hidden variables but we ignore such dependence here.) The vector $\mathbf{r}$ is expressed by its elements:

$$\mathbf{r}=\begin{bmatrix} r_1 \\ r_2 \\ \cdot \\ \cdot \\ r_R \end{bmatrix}$$

The r_j are so defined that the rates of production (moles per unit time per unit volume of culture) of any chemical species A_i are given by

$$\sum_{j=1}^{R} \alpha_{ij}r_j (i=1, 2, \ldots, S).$$

It is convenient to define two matrices $\boldsymbol{\beta}$ and $\boldsymbol{\gamma}$ by

$$\boldsymbol{\beta}=\begin{bmatrix} \alpha_{11} & \alpha_{12} & \cdots & \alpha_{1R} \\ \alpha_{21} & \alpha_{22} & \cdots & \alpha_{2R} \\ \cdot & \cdot & & \cdot \\ \cdot & \cdot & & \cdot \\ \cdot & \cdot & & \cdot \\ \alpha_{I1} & \alpha_{I2} & \cdots & \alpha_{IR} \end{bmatrix}, \qquad (3a)$$

$$\boldsymbol{\gamma}=\begin{bmatrix} \alpha_{I+1,1} & \alpha_{I+1,2} & \cdots & \alpha_{I+1,R} \\ \alpha_{I+2,1} & \alpha_{I+2,2} & \cdots & \alpha_{I+2,R} \\ \cdot & \cdot & & \cdot \\ \cdot & \cdot & & \cdot \\ \cdot & \cdot & & \cdot \\ \alpha_{S1} & \alpha_{S2} & \cdots & \alpha_{SR} \end{bmatrix}. \qquad (3b)$$

These are called stoichiometric matrices, the first for materials in the biological phase, the second for materials in the liquid phase.

The set of quantities embodied in the stoichiometric matrices, $\boldsymbol{\beta}$ and $\boldsymbol{\gamma}$, and in the

vector of reaction rates, $\mathbf{r}$, are the parameters that we have chosen for the expression of the general, non-segregated model. They have well defined physical and biological meaning and are measurable; the fact that they appear in a model means that the model is an expression of postulated cause-and-effect relationships. That is, the quantities in β, γ, and $\mathbf{r}$ have a significance beyond that of quantities determined simply to make an equation fit experimental data.

Much physiological research on a given organism is intended to answer these questions:

What intracellular reactions occur, and what is their stoichiometry (i.e., what are the α_{ij})?

What are the rates of those reactions, and how do they depend on internal and external factors (i.e., how do the r_j depend on the vectors $\mathbf{x}$ and $\mathbf{c}$)?

From the answers we can construct specific models and predict the dynamical behavior of a culture of the organism by applying the equations that will now be described.

DYNAMICAL EQUATIONS FOR THE CONTINUOUS PROPAGATOR

Application of the principles of mass conservation and stoichiometry to the various biochemical species present in the biological phase of the culture yields the matrix equation

$$\frac{d\mathbf{x}}{dt}=-D\mathbf{x}+\beta\cdot\mathbf{r}, \tag{4}$$

where D is the so-called "dilution rate" and equals the volumetric flow rate of fresh medium divided by the volume of culture in the vessel. The reciprocal of D is the "holding time." The term on the left-hand side of the equation represents the rates of accumulation of biochemical species in the biological phase; the first term on the right represents the rates of washout with the effluent culture, and the last term on the right represents the rates of production by intracellular and transfer reactions.

Application of the principles of mass conservation, stoichiometry, and interfacial mass transfer to the various chemical species in the liquid phase of the culture yields the matrix equation

$$\frac{d\mathbf{c}}{dt}=D(\mathbf{c}^f-\mathbf{c})+\gamma\cdot\mathbf{r}+\frac{1}{1-\emptyset}\,\mathbf{K}\cdot(\mathbf{c}^*-\mathbf{c}) \tag{5}$$

Herein, $\mathbf{c}^f$ is the vector of concentration in the (liquid) feed stream, $\mathbf{K}$ is the matrix of overall mass transfer coefficients, and $\mathbf{c}^*$ is a vector representing the composition of a liquid phase in thermodynamic equilibrium with the gas phase. The various terms in the equation represent, reading from left to right: accumulation in the liquid phase, net input due to flow of liquid through the culture vessel, production by transfer reactions, and input by transfer from the gas phase.

We could apply the same principles to yield a differential equation for $\mathbf{p}$, the vector of partial pressures in the gas phase, but this would introduce needless complications. Therefore, we simply assume that the composition and pressure of the gas phase is constant, so that $\mathbf{c}^*$ is a constant vector.

Thus, equations 4 and 5 are the dynamical equations of the propagator. If we knew (1) the stoichiometric matrices β and γ, (2) the dependence of the reaction-rate vector $\mathbf{r}$ on vectors $\mathbf{x}$ and $\mathbf{c}$ and (3) the matrix of overall transfer coefficients, $\mathbf{K}$, then the equations could be solved numerically to predict the time course of development (the dynamics) of the propagator. In practice, this information is not available; we seek instead to use the observed dynamics to establish β, γ, $\mathbf{r}$, and $\mathbf{K}$.

BALANCED GROWTH

Campbell (ref. 10) introduced the important concept of balanced growth. Later, Lamanna and Mallette (ref. 11) defined the concepts of restricted and unrestricted balanced growth. Since these concepts have importance in continuous propagation, we shall translate the definitions of Campbell and Lamanna and Mallette into mathematical terms.

Let us define a set of quantities μ_i ($i=1,2,\ldots,I$) by the equation

$$\mu_i=\frac{1}{x_i}\sum_{j=1}^{R}\beta_{ij}r_j. \tag{6}$$

Since the sum represents the rate of production of the i^{th} component of the biological phase per unit volume of culture, it is clear that μ_i is the same rate of production, but per unit amount of the i^{th} component of the biological phase. Hence, the μ_i are called the specific replication rates. Equation 4 may then be rewritten in component form as

$$\frac{dx_i}{dt} = -Dx_i + \mu_i x_i. \quad (i=1, 2, \ldots, I). \qquad (7)$$

According to Campbell (ref. 11), "... growth is balanced over a time interval if, during that interval, every extensive property of the growing system [such as the x_i] increases by the same factor." Hence, if the concentration of the i^{th} constituent of the biological phase changes from x_i to x_i+dx_i during an infinitesimal time interval, then growth will be balanced during that time interval, provided that

$$\frac{dx_i}{x_i} = \frac{dx_k}{x_k} \quad (i, k=1, 2, \ldots, I).$$

Equation 7 then shows that the conditions for balanced growth are

$$\mu_1 = \mu_2 = \ldots = \mu_I. \qquad (8)$$

It can also be shown that in balanced growth the composition of the biological phase does not change with time. Growth can be balanced only in populations in which individual cell fissions occur at random intervals. That is, growth cannot be balanced if there is an appreciable synchrony in cell division. A more general kind of situation, called repetitive growth, has been considered by Fredrickson, Ramkrishna, and Tsuchiya (ref. 5). This situation includes balanced growth and synchronous growth (repetitive growth is outside the scope of this paper).

Growth in the continuous propagator is balanced if the propagator is operating in the steady state. For, if operation is steady, $d\mathbf{x}/dt=\mathbf{0}$ (and also $d\mathbf{c}/dt=\mathbf{0}$), and equation 7 reduces to $D=\mu_i$, which implies equation 8.

Growth in a batch propagator ($D=0$) may be balanced. We have not developed here the apparatus necessary to state which conditions guarantee balanced growth in batch propagation. This problem has been considered by Fredrickson, Ramkrishna, and Tsuchiya (ref. 5), and it has been shown that batch propagation is balanced growth if the population density increases exponentially and the specific replication rates (the μ_i) are independent of environmental conditions, **c**. If, in fact, the μ_i are independent of **c**, then internal cellular factors limit the rate of growth, and Lamanna and Mallette (ref. 11) call the situation unrestricted balanced growth. That is, the growth rate is unrestricted by environmental conditions. Obviously, balanced growth, whether restricted or unrestricted, is in some sense a steady-state situation, in which cells have adjusted themselves to their environment. In continuous propagation, it is a completely steady-state situation, in that all quantities **x, c, p** are independent of time. In batch-balanced growth, the situation is steady in the sense that the composition of the biological phase (and hence the physiological activities of the cells) is independent of time. Thus, balanced growth is very convenient for experimental exploitation. For instance, if one has a model, the constants of the model might be measured most readily by balanced-growth experiments. In order to test the model, its predictions could be compared with observations made on experimental situations other than balanced growth.

COMPARISON OF CHEMOSTAT AND TURBIDOSTAT TYPES OF OPERATION

Ideally, one could operate a turbidostat in unrestricted balanced growth. This possibility rests on the observation that rates of many cellular processes exhibit no dependence on environmental conditions over certain ranges of those conditions. Thus, growth rates exhibit "saturation phenomena"; that is, growth rates are independent of substrate concentrations if those concentrations are sufficiently high (but not too high). Again, synthesis rates may exhibit "threshold phenomena" with respect to the action of inhibitory substances; that is, synthesis rates are independent of inhibitor concentrations if these concentrations are low enough. To obtain unrestricted balanced growth in a continuous propagator, we use a growth medium with high substrate concentrations and low inhibitor concentrations. We maintain satura-

tion levels of dissolved gases by using sufficient agitation and a sufficiently high gas concentration. We combine these with control of the flow rate via culture density so that concentrations of substrates and inhibitors are above the saturation levels and below the threshold levels, respectively. If in fact the turbidostat is operating in unrestricted balanced growth, then the material balance equations on the liquid phase of the culture and the gas phase become irrelevant to the dynamical behavior of the propagator; the dynamical behavior is governed now by the single set of equations

$$\frac{d\mathbf{x}}{dt} = -D\mathbf{x} + \boldsymbol{\beta} \cdot \mathbf{r}$$

where D depends on the generalized culture density, y, according to the kind of control adopted, and $\tilde{r} = \tilde{r}(\mathbf{x})$ represents the vector of reaction rates when substrates are present in saturating concentrations and inhibitors are present in sub-threshold concentrations.

It is sometimes stated that because the turbidostat operates in unrestricted balanced growth, the growth rate therein must be the maximum attainable at a given temperature. In particular, it is stated that the growth rate in the turbidostat is necessarily greater than that obtainable in the chemostat. This statement is not theoretically correct (though it may be correct in certain practical cases) because it fails to take into account the possibility of adaptation of the biomass. There is no theoretical reason why an organism could not be trained to grow at a faster rate in a chemostat situation than it does in a turbidostat situation. Of course, growth will be faster in the turbidostat than in the chemostat if the training of the organisms is the same in both situations.

Operation of the turbidostat in unrestricted balanced growth limits the obtainable amount of information concerning cellular kinetics. Obviously, we want to know how the cellular reactions depend on environmental conditions, and this is precisely the information that cannot be obtained in unrestricted balanced growth. In addition, if the turbidostat principle is to be used for life-support applications, limitations on the concentrations of substrates in the feed may place quite severe restrictions on the level of culture density that can be used.

For these reasons, it is often advantageous to operate the turbidostat in a state of restricted balanced growth. To do so introduces the possibility of an interesting dynamical effect, which must be mentioned here. Figure 2 shows a typical plot of culture density vs dilution rate for steady-state operation of the propagator as a chemostat, i.e., operation in restricted balanced growth with a time-independent dilution rate. This curve is for a particular feed composition ($\mathbf{c}^f$); changing it would give a different curve. The curve is typical of many organisms, including bacteria (ref. 12) and yeasts (ref. 13). The features of the curve are so well-known that it is only necessary to point out (1) that the decline of the curve at low dilution rates is due, at least in part, to endogeneous metabolism, and (2) that at high dilution rates, cells are physiologically "young" whereas as the dilution rate is lowered, cells become progressively "older."

The curve will also describe the steady-state operation of the turbidostat, if that is operating in restricted balanced growth. Suppose the controller for flow rate is such that culture density is to be maintained at a level y_0. The aim of the control is to operate at steady state A. At the high dilution rate D_A corresponding to this steady state, cells are physiologically young, and the turbidostat operates just out of the range of unrestricted balanced growth—the dashed extension of the curve. However, there is another possible steady state B, corresponding to a culture density y_0. At this

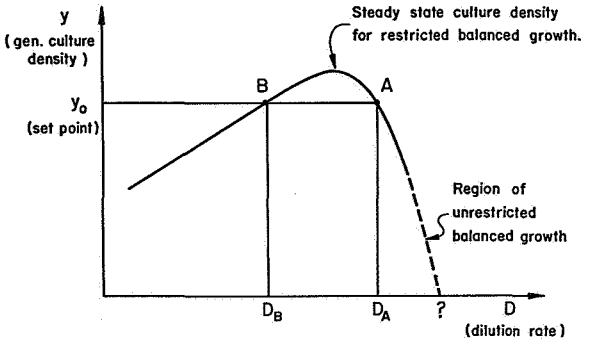

FIGURE 2.—Steady-state relations in continuous microbial propagation.

steady state, the dilution rate is much lower, D_B, and cells are physiologically much older than cells corresponding to steady state A.

Note that a controller actuated by culture density alone cannot distinguish between steady states A and B. Moreover, both steady states may be dynamically stable, in the sense that small perturbations from them would decay with time. Hence, which steady state the propagator would operate in depends on how the culture is started up. Or—even more important from a practical point of view—the steady states would not necessarily be stable to large perturbations, so that a large perturbation from the desired steady state, say A, might cause the propagator to seek the other steady state, B. There would then be a very serious deviation of the metabolic activities of the culture from those desired. Large perturbations in continuous propagators could result from a variety of causes, such as a pump or power failure, alteration of the composition of the liquid nutrient feed, failure of the gas supply, or changes in temperature. Hence, it seems desirable to see if switches of steady states are a practical possibility in any proposed turbidostat propagators.

It is not suggested that the foregoing phenomena necessarily happen in turbidostat operation. Possibly, the shape of the y-vs-D curve and the magnitude of likely perturbations will prevent that switching to an undesired steady state. But such switching can occur.

In chemostat-type operation, switching is not possible, because there is only one steady state corresponding to a given flow rate. However, large perturbations from the steady state may lead to washout, unless the dilution rate is temporarily lowered.

In conclusion, note that in either the chemostat or the turbidostat there may be no true steady state. Internal feedback mechanisms in the biological phase (or external feedback also, in the turbidostat) may produce sustained oscillations in the propagator. An example of this is presented in the next part of this paper.

NOTATION

A_i	Denotes the i^{th} chemical species.
$\mathbf{c}$	Vector of concentrations in the liquid (elements c_i).
$\mathbf{c}^f$	Vector of concentrations in the liquid feed.
c_i'	$c_i/(1-\phi)$.
$\mathbf{c}^*$	Vector of concentrations of liquid in equilibrium with gas phase (elements c_l^*).
D	Dilution rate.
I	Number of chemical species in the biological phase.
$\mathbf{K}$	Matrix of overall mass-transfer coefficients (elements K_{il}).
$\mathbf{m}$	Vector of weight factors for the generalized culture density (elements m_i).
$\mathbf{p}$	Vector of partial pressures in the gas phase.
R	Number of chemical reactions (intracellular and transfer).
$\mathbf{r}$	Vector of reaction rates (elements r_j).
$\tilde{\mathbf{r}}$	Vector of reaction rates in unrestricted balanced growth.
S	Number of chemical species in liquid phase.
t	Time.
$\mathbf{x}$	Vector of concentration of biochemicals (elements x_i).
y	Generalized culture density.
α_{ij}	Stoichiometric coefficient of A_i in j^{th} reaction.
$\boldsymbol{\beta}$	Stoichiometric matrix for cellular chemical species (elements β_{ij}).
$\boldsymbol{\gamma}$	Stoichiometric matrix for environmental chemical species (elements γ_{ij}).
μ_i	Specific replication rate of A_i.
ϕ	Volume fraction of biological phase in culture.

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COMMENTS

Dr. JENKINS. When you speak of restricted steady state and turbidostat, are you talking about limiting the growth by having one factor set at a low level?

Dr. FREDRICKSON. One or more factors, yes.

Dr. COOKE. What do you call a chemostat?

Dr. FREDRICKSON. The chemostat is the case where the flow rate of fresh liquid to the propagator is constant. There is no control on it. With the turbidostat, we monitor the culture density and control the flow rate in terms of culture density. If the culture density gets too high, the controller says: feed faster. If it gets too low, the controller says: feed slower.

Dr. COOKE. Can you ever obtain a steady-state growth with the chemostat?

Dr. FREDRICKSON. Yes.

Dr. COOKE. How? Isn't it always going either way? I cannot see how you can make a stable condition with the chemostat. Does that work?

Dr. FREDRICKSON. Yes.

Dr. TSUCHIYA. In the turbidostat, you have sensors which monitor—as John Foster is doing—nitrogen or whatever and puts in some medium, whereas in the case of the chemostat, you set the flow rate yourself. Therefore, the control now has some limiting substrate.

Dr. COOKE. You can still have good culture?

Dr. TSUCHIYA. This is up to the experimenter.

Dr. COOKE. What can you use in principle to limit the growth?

Dr. TSUCHIYA. We will have an example of that later. We know what is required insofar as organisms are concerned for them to propagate. In our case, we have chosen glucose, which we shall discuss.

Dr. FREDRICKSON. In the chemostat, you can choose anything that you wish to be limiting by what you choose for the composition of the feed medium.

Dr. COOKE. But if you want to get optimal growth, if your goal is to get the most for your money, what can you gain from a chemostat? I know you can use it for studying several things but, to study optimal yield per weight, what can you learn from the chemostat?

Dr. WARD. You must have restricted conditions.

Dr. FREDRICKSON. In the chemostat, yes, but in the turbidostat you can have unrestricted balanced growth. The point is that in most turbidostat operations, you do not have unrestricted balanced growth, because this is a restricted situation and there is not a very large range of conditions that will give you unrestricted balanced growth. The point of using the chemostat is, for one thing, that you can gain information about what is happening. You can find out how the reaction rates depend upon the applied conditions; in other words, how the reaction rates depend upon the vector I called C, the concentrations in the environmental conditions. If you operate a turbidostat in unrestricted balanced growth, the reaction rates do not depend upon the external conditions. That is what is meant by unrestricted balanced growth. You get no information from such an experiment as to the variation of the reaction rates with changing external conditions.

Dr. COOKE. I don't see that you get useful information.

Dr. FREDRICKSON. Certainly, you get information about the kinetics of growth.

Dr. COOKE. I don't see how you get kinetics out of this.

Dr. BONGERS. To define kinetic growth, the organ has to be supported by its environment. Let us say you would look at temperature, for instance. What you are trying to describe is the effect of temperature on enzyme systems.

Dr. FREDRICKSON. I said nothing about temperature, but you could do that if you wanted to.

Dr. BONGERS. Let us say we flow medium in at a given rate. You have to define your medium.

Dr. FREDRICKSON. Yes.

Dr. BONGERS. You have to define one factor. You have to study one factor of the environment which finally gives you maximal growth.

Dr. FREDRICKSON. I don't see the point of what you are saying.

Dr. TSUCHIYA. You have to look at the medium composition, is that correct?

Dr. BONGERS. No. All I am saying is that there was always one factor that determined your rate in all cases.

Dr. FREDRICKSON. Something determines the rate in all cases, yes. Whether it is one single factor, I would not want to say.

Dr. BONGERS. One enzyme system, I would say, which determines your rate, finally.

Dr. COOKE. I cannot see how a chemostat will answer your question in this case. You know that if you do not feed glucose or hydrogen, it will not grow. What do you learn from there on?

Dr. FREDRICKSON. For one thing, you could certainly vary the environmental conditions. You are not constrained to feed the same medium. You also do not have to operate in balanced growth. You can study transients in order to learn something about the kinetics. In fact, this is one of the things that Dr. Tsuchiya is going to talk about—how you might learn something about the kinetics from the transients you observe.

Dr. TISCHER. In one of your first figures, you mentioned x_i and c_i measuring composition. Is this meant to be growth composition, cationic composition, and anionic, or what?

Dr. FREDRICKSON. The x_i's represent the molecular components of the biological phase. The c_i's represent all of the components which are present in the liquid phase, so this would be cationic, anionic, unionized species.

Dr. TISCHER. What do you do in your models about the practical situation surrounding the use of concentrations, let us say, in place of activities or fugacities?

Dr. FREDRICKSON. I have not said that I am using concentrations rather than fugacities or activities, because fugacities are functions of concentration.

Dr. TISCHER. So is activity.

Dr. FREDRICKSON. Yes. In the simplest case where concentrations are low, then concentration and activity become synonymous. Even if concentrations are not low. If you tell me what the concentration is, I can still tell you what the activity is. It was in that sense that I used the concentrations rather than the activities here. But for practical purposes, I express driving forces in terms of concentrations rather than activities, because otherwise it gets to be such a mess.

Dr. TISCHER. You mentioned constant composition. I presume you mean dynamically constant composition and not statistically constant?

Dr. FREDRICKSON. I mean the relative composition of the biologic phase. If growth is balanced, and you determine the relative composition at two different instances of time, these compositions must be the same. Otherwise, you would not have balanced growth.

Dr. TISCHER. How do your models, if that is the case, account for interactive effects of two or more factors up through end factors? I am talking about statistically defined interaction, in which activity one in conjunction with activity two gives a greater response than the sum of the two activated separately.

Dr. FREDRICKSON. Again, I think you have to look at a specific example. All I will say is that the model is general enough to account for such interactions. Precisely how it is done depends upon the specific model that you are talking about, and Dr. Tsuchiya will have an example.

Dr. TISCHER. What I was thinking of, for instance, is if attribute x and c, to put it generally, are not completely orthogonal, they must interact. Therefore, if you operate these two items in the same mixture, the result is not going to be a completely additive one. It is going to be something more than that. I did not see anything in your vector matrices or your square rectangular arrays which specifically accounted for this. It left me behind on that score.

Dr. FREDRICKSON. I did not want to talk about a specific model. All I am saying is that such effects can be accounted for in terms of the reaction rates.

Dr. KOK. On one curve you had two cross points which I did not quite get. Is it not true that in actual culture you would use only the maximum of these curves? That would be your optimal growth? Your criticism does not apply—or am I mistaken in that order?

Dr. FREDRICKSON. Not quite. You would have to multiply or divide this by a factor of d. I do not remember which.

Dr. KOK. Then you would come out at the top, most likely?

Dr. FREDRICKSON. Not necessarily. It depends on what you try to optimize. If you want to optimize production of cells per unit volume, then it would all right. If you want to optimize the concentration, then you would operate at another point on the curve. But that may not necessarily be what you want to optimize. Maybe you want to optimize the rate of some metabolic process, and maybe that would say you should be operating elsewhere on the curve.

Dr. KOK. In the particular terms of this graph, does the maximum coincide with maximum production or not? I am not sure. It certainly is the minimum rate, but also the maximum production.

Dr. FREDRICKSON. I think it would be close to this point, but would not necessarily be the same. I am not sure.

Dr. MILLER. Would not the production rate be the product y times d, and you would want to plot y times d against d on the abscissa; the maximum would probably fall slightly to the right of the maximum population density.

Dr. FREDRICKSON. Yes. The curves would be similar in shape. They would not necessarily have the same maximum.

PHOTOSYNTHESIS—ALGAL SYSTEMS

C. H. WARD, *Chairman*

The Food Chain

J. F. Drake, J. L. Jost,
A. G. Fredrickson, and H. M. Tsuchiya
University of Minnesota
Minneapolis, Minnesota

Much is known qualitatively concerning various food chains on earth but very little concerning the processes whereby proteins of the prey are upgraded by the predator. Lotka and Volterra (ref. 1) formulated a mathematical relationship between population densities in a food chain. A set of modified Lotka-Volterra equations representing the relationship between predator and prey populations is:

$$\underset{\text{accumulation rate}}{\frac{dN_1}{dt}} = \underset{\text{growth rate of } N_1}{aN_1} - \underset{\text{predation rate of } N_1 \text{ by } N_2}{bN_1N_2} - \underset{\text{washout rate}}{\frac{N_1}{\theta}}$$

$$\underset{\text{accumulation rate}}{\frac{dN_2}{dt}} = \underset{\text{growth rate of } N_2}{cN_1N_2} - \underset{\text{death rate of } N_2}{dN_2} - \underset{\text{washout rate}}{\frac{N_2}{\theta}}$$

where

N_1 is number of prey
N_2 is number of predators
a,b,c,d are constants
θ is holding time
Q is feed rate
V is volume.

Let $D=\frac{Q}{V}=\frac{1}{\theta}$.

Lotka and Volterra's equations have been modified here to apply to continuous as well as batch cases; in the latter, $\theta = \infty$. These equations do not take into account the environment, which influences the prey population and hence the other reaction rates. Moreover, the constants a, b, c, d are not broken down to the point where they might be useful in predictions. The growth terms as used by Monod (ref. 2) and Herbert et al. (ref. 3), and others, and death terms as proposed by us (ref. 4) could be substituted for interaction terms in these equations.

Contois and Yango (ref. 5) have reported on the *Dictyostelium discoideum—Aerobacter aerogenes* system of "pure" mixed cultures. These investigators followed changes in predator population density, but unfortunately did not measure prey density nor the concentration of limiting substrate for the prey. Under their experimental conditions, with a nitrogen-limiting medium for bacteria, they observed no oscillations when θ was 6.7 and 16.7 hours (i.e, D was 0.15 and 0.06 hr^{-1}, respectively) over the week in which they observed their culture. This is a report of a system of *D. discoideum* (a myxameba) and *Escherichia coli*, in which populations of the two were grown in a simple glucose, ammonium-sulfate, salts medium; glucose was limiting for *E. coli*. We discuss first our analytical model and then some data on densities of the two populations and glucose.

MODEL

Equations similar to those of Monod (ref. 2), Herbert et al. (ref. 3), and ourselves (ref. 4) were developed to establish the interactions be-

tween the predator (*D. discoideum*), the prey (*E. coli*), and glucose (the limiting substrate for *E. coli*). The population density of *D. discoideum* depends primarily on the availability of *E. coli;* the density of *E. coli* depends on the limiting supply of glucose and on the predation by *D. discoideum.*

The predator undergoes a life cycle—spore, ameboid or vegetative stage, pseudoplasmodium, and culminatory stages (ref. 6) under the cultural conditions usually employed in its study. Our experiments were conducted in continuous submerged culture in a Novick-Szilard chemostat (ref. 7). Under such conditions, the predator occurs only in the ameboid stage so "predator" and "ameba" are synonymous. Note that the ameba (but not the prey) cannot use glucose.

The following reactions represent growths of the bacteria and ameba

1. $B+a_sS\longrightarrow 2B+\text{------}$
2. $A+a_bB\longrightarrow 2A+\text{------}$
3. $B+a'_aN_A\longrightarrow 2B+\text{------}$
4. $A+a'_bN_B\longrightarrow 2A+\text{------}$
5. $A+a''_bA\longrightarrow 2A+\text{------},$

where

a's are stoichiometric coefficients,
N's are "dead" organisms.

Reactions 1 and 3 represent the growth of *E. coli* on glucose and dead ameboid material, respectively. Reactions 2, 4, and 5 represent the predation of ameba on live *E. coli*, dead *E. coli*, and one another (cannibalism), respectively. Raper (ref. 8) and Bonner (ref. 9) have reported on the feeding of ameba by dead bacteria and by cannibalism, respectively.

Both organisms need enegy to maintain structure and function, and to grow. All organisms die; the mechanism of death assumed here was that predators of our systems die of starvation and (in the case of *E. coli*) by predation only. These additional considerations may be summarized by a model of Ramkrishna et al. (ref. 4) which is comparable to the "activated" complex theory of chemical kinetics (ref. 10). Here the activated complex is a form of organism in a physiological state different from that of the "normal" or "dead" organism. We call this the "degenerate form," resulting from starvation. This form can either revert back to a normal organism or die. Thus the death reactions would be:

6. $A\longrightarrow A'\rightarrow N_A$
7. $B\longrightarrow B'\rightarrow N_B,$

where A', B' are degenerate forms.

The back reactions whereby the degenerate forms can revert back to normal organisms would be:

8. $A'+a'_bB\rightarrow A$
9. $B'+a'_sS\rightarrow B.$

We assume that degenerate forms of ameba and bacteria return to normal forms by feeding upon bacteria or glucose, respectively.

We now make some simplifying assumptions. Observations have shown that bacteria are not likely to die of starvation very much, under our experimental conditions. Predation was assumed to be the only mechanism of bacteria death; thus we ignore reactions involving starvation. Similarly we ignore the maintenance reaction of bacteria (this may be an oversimplifying assumption). Because the ratio C_B/C_A was usually large (about three orders of magnitude), reaction 5 was neglected. Finally, we assumed that bacteria do not feed on materials from dead ameba, reaction 3.

These assumptions leave the following reactions whose rates must be considered:

1. $B+a_sS\longrightarrow 2B+\text{------}$
2. $A+a_bB\longrightarrow 2A+\text{------}$
6. $A\rightarrow A'\longrightarrow N_A$
8. $A'+a'_bB\longrightarrow A.$

Rates of the growth reactions (1 and 2) are expressed in the Michaelis-Menten form:

$$r_1=\frac{\mu_B C_B C_S}{C_S+K_S};\ r_2=\frac{\mu_A C_A C_S}{C_S+K_S}$$

where

K_S, K_B are saturation constants,
μ_A, μ_B are maximal specific growth rates,
C_S, C_A, C_B are concentrations.

From these assumptions the mathematical consequences were developed. Our equations differ from those of Lotka and Volterra, which assumed that the dynamical behavior of the

populations of the predator and prey depend solely on the population densities and on the lump parameter constants; this does not account explicitly for some of the environmental factors. Our equation takes explicit account of the dependence of prey population on substrate concentration and ameba predation; also the dependence of the predator population on prey population and death due to starvation. Although the equations apply to both batch and continuous cultures, most of our work dealt with the latter. A system of differential equations was derived (making some simplifying assumptions).

$$\frac{dC_A}{dt} = \frac{\mu_A C_A C_B}{K_B + C_B} - \frac{\beta_A C_A}{K'_b + C_B} - \frac{C_A}{\theta}$$

accumulation rate | predator growth rate | predator death rate | washout rate

$$\frac{dC_B}{dt} = \frac{\mu_B C_B C_S}{K_S + C_S} - \frac{a_b \mu_A C_A C_B}{K_B + C_B} - \frac{a'_b \beta'_A C_A C_B}{K'_B + C_B} - \frac{C_B}{\theta}$$

accumulation rate | prey growth term | predation rate | predator maintenance rate | washout rate

$$\frac{dC_S}{dt} = -\frac{a_S \mu_B C_B C_S}{K_S + C_S} + \frac{C_{S_f} - C_S}{\theta}$$

accumulation rate | substrate utilization rate | washout rate

where

C's are concentrations
μ's are maximal specific growth rates
K's are saturation rates
a's, 's are stoichiometric coefficients.

The various constants were determined experimentally in batch cultures. For the predator, μ_A was taken as $\Delta \ln C_A/\Delta t$; K_B as the bacterial concentration at $\frac{1}{2}\mu_A$; a_b as $\Delta C_B/\Delta C_A$; all measured during exponential growth in batch cultures of mixed populations without glucose. For the prey, the constants μ_B, K_S, and a_S were obtained in similar manner in batch cultures of *E. coli* with glucose. The constants β_A, $a'_b\beta'_a$, and K'_b were obtained from batch cultures of mixed populations with glucose and evaluated by the quasi-linearization method of Bellman (ref. 11).

The experimentally determined constants are summarized as follows:

μ_A, (hr^{-1})	a_b, $\left(\frac{no.}{no.}, \times 10^{-3}\right)$	K_B, $\left(\frac{no.}{ml}, \times 10^{-8}\right)$
0.24	1.8	2.5
μ_B, (hr^{-1})	a_s, $\left(\frac{mg.}{no.}, \times 10^{10}\right)$	K_s, $\left(\frac{mg.}{ml}, \times 10^{4}\right)$
0.60	5.1	5.0
β_A, $\left(\frac{no.}{hr}, \times 10^{-10}\right)$	$a'_b\beta_A$, $\left(hr^{-1}\frac{no.}{no.}\right)$	K'_B, $\left(\frac{no.}{ml}, \times 10^{-8}\right)$
1.6	74.0	3.0

The constants in these equations depend upon the organisms, temperature, pH, medium constituents, and other environmental factors.

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The constants in these equations depend upon the organisms, temperature, pH, medium constituents, and other environmental factors.

EXPERIMENTAL

All experiments (except the batch ameba-bacteria experiments in which no glucose was present) were conducted in a glucose, ammonium-sulfate, and salts medium with an initial pH of 7.0–7.1 (table I). Glucose was sterilized separately and added at levels of 0.01 to 0.5 percent. The yield of bacteria is proportional to the sugar concentration, over the lower portion of this range. The majority of continuous-culture experiments were carried out at 0.5-percent level where glucose is limiting.

TABLE I.—*Composition of Medium, Continuous Culture*

Item	gm/l
K_2HPO_4	3. 676
KH_2PO_4	1. 50
$MgSO_4.7H_2O$	0. 100
NaCl	0. 010
$(NH_4)_2SO_4$	1. 25
Glucose	5. 00

Batch cultures were run in either 300 or 1000 ml Erlemeyer flasks containing 100 or 300 ml, respectively, of media at 24°C and on a reciprocating shaker. The *E. coli* cultures were sampled hourly; the ameba cultures were sampled every four to eight hours. Continuous cultures were grown in Novick-Szilard chemostats and were sampled at least every eight hours over periods of weeks. Population densities were determined with a Coulter counter after appropriate dilutions in 0.6-percent saline. Cell-size distributions for *E. coli* were recorded with a Coulter-size distribution plotter used in conjunction with the counter. Both the saline solution (used for dilution) and the sterilized media required filtration through 0.1-micron millipore filter to reduce background count and prevent clogging of the 30-micron aperture (used for bacteria). Glucose determinations were made by the glucostat method (ref. 12). The samples were filtered immediately through 0.45-micron millipore filters; if analyses were to be delayed, the samples were quickly frozen.

RESULTS

Oscillations in predator and prey population densities and concentrations of glucose were observed for some two weeks; then an apparently pseudo-steady state was attained when Θ was 14.4 hours (dilution rate of 0.0685 hrs^{-1}) and at 24°C.

The numbers of the ameba oscillated sevenfold, between 1×10^5 to 7×10^5 per ml. The bacterial density oscillated some 40-fold from 0.3×10^8 to 13.0×10^8 per ml. The peak of ameba density occurred out of phase with and slightly after the peak of the bacterial density (fig. 1). The peak of the bacterial density was almost 180 degrees out of phase with and slightly lagged behind the glucose concentration (fig. 2).

The apparent attainment of a pseudo-steady state is hard to explain. Did the organisms "adapt"? The possibility of contamination is not

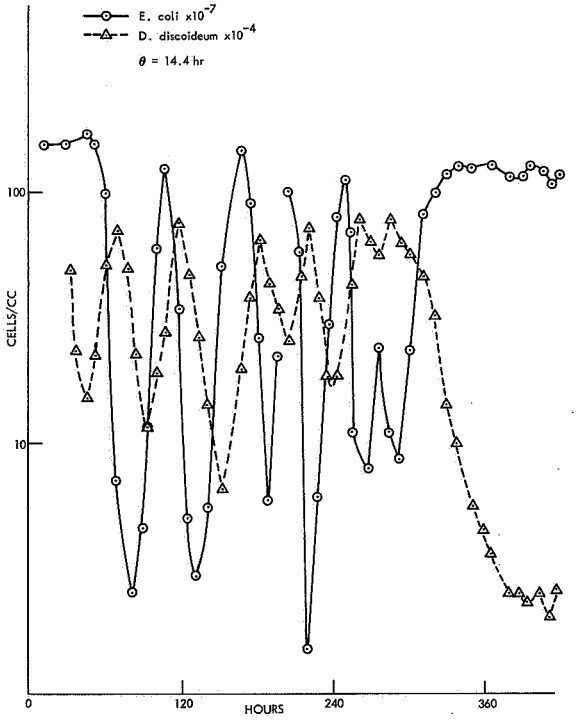

FIGURE 1.—Ameba and bacterial density vs time, at 24°C, Θ=14.4 hr.

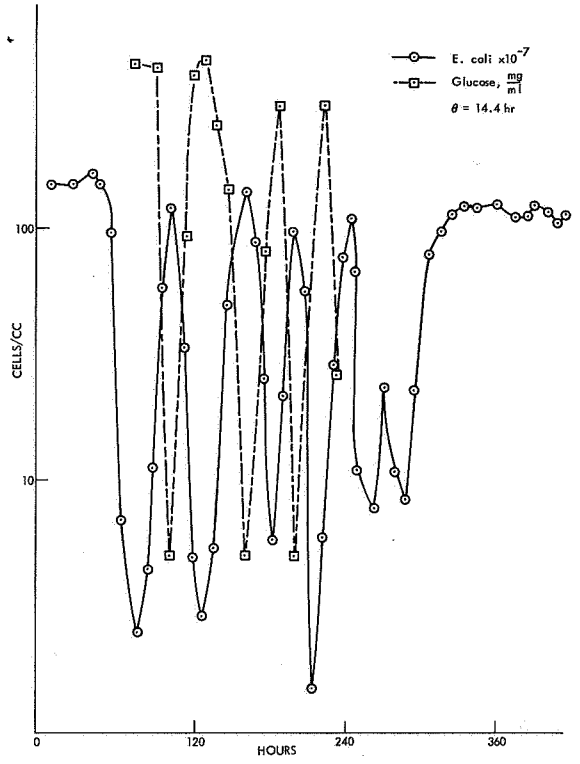

FIGURE 2.—Bacterial density and glucose concentration vs time at, 24°C, Θ=14.4 hr.

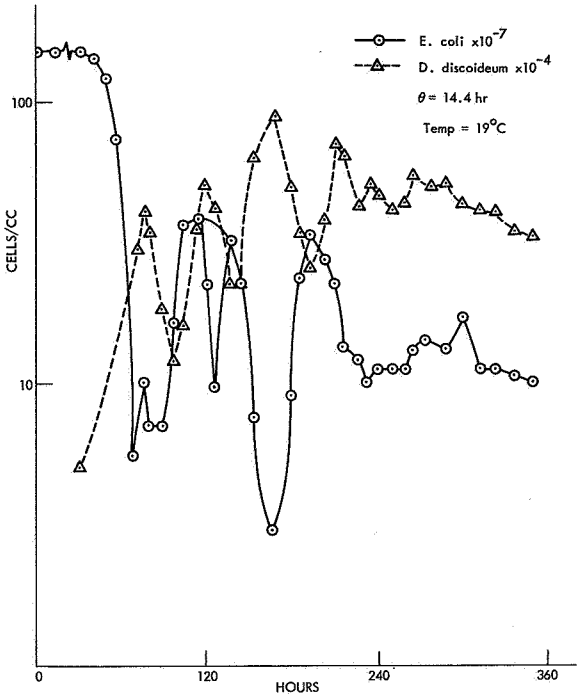

FIGURE 3.—Ameba and bacterial density vs time, at 19°C, Θ=14.4 hr.

ruled out, though checks were made daily. This question demands further inquiry.

When the temperature was lowered to 19°C but Θ was held constant, oscillations were again observed. The numbers of ameba oscillated as much as at 24°C but the bacterial oscillated only fourfold. At this temperature the μ's and K's should be about the same as at 24°C for the ameba but lower for *E. coli*. The stoichiometric constants would probably not vary too much (See figs. 3 and 4.)

Over a given oscillation, the environment changes. The variation would be reflected in cytological properties of predator and prey. At or close to their maximal density, the bacteria were nutrient-limited and small, as seen by the size distribution (fig. 5). As predation lowered their numbers, glucose accumulated. We would expect a lag in bacterial growth because organisms at or near the peak were probably similar physiologically to cells in the stationary phase in batch cultures (refs. 13, 14). On the other hand, at or close to their minimal density, the bacteria

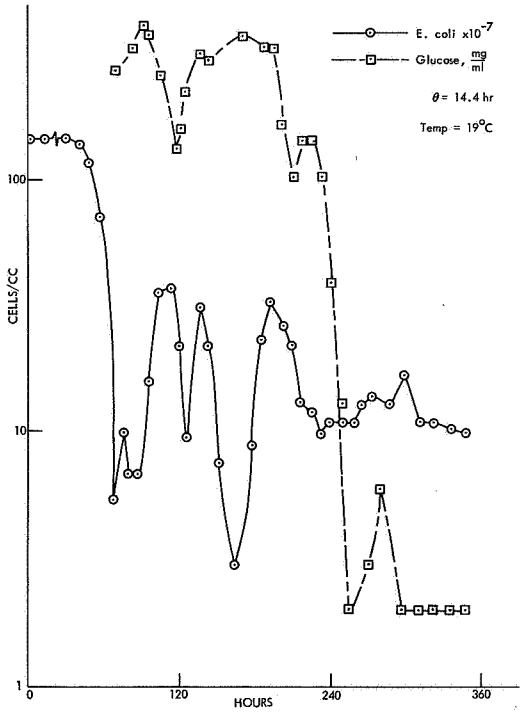

FIGURE 4.—Bacterial density and glucose concentration vs time, at 19°C, Θ=14.4 hr.

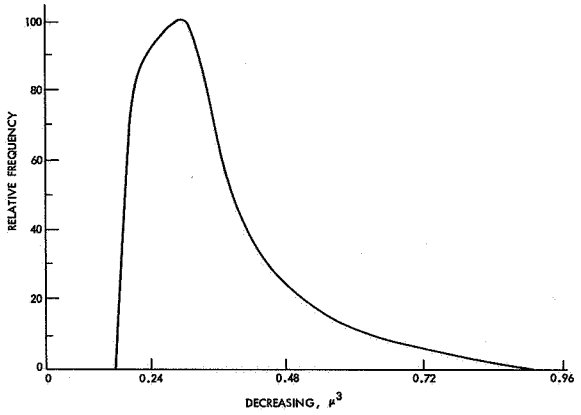

FIGURE 5.—Bacteria size distribution (decreasing).

increase in size and grow rapidly. The size distribution (fig. 6) is broad with a higher mean size. The considerable numbers of very large cells causes "tailing-out" of the distribution at its upper end.

Comparable size distributions of the ameba could not be measured, because the samples taken were too small (the chemostat tube volume was approximately 26.5 ml). On the other hand, microscopic examination disclosed gross morphological changes. Ameba at or near their maximal density were round, hyaline, vacuolated, and readily lysed by contrast, rapidly growing ameba at or near their minima were turgid and showed considerable pseudopodial activity and a random "convective" type of protoplasmic activity.

DISCUSSION

The differential equations, using the experimentally determined constants were numerically integrated on a digital computer. When θ was

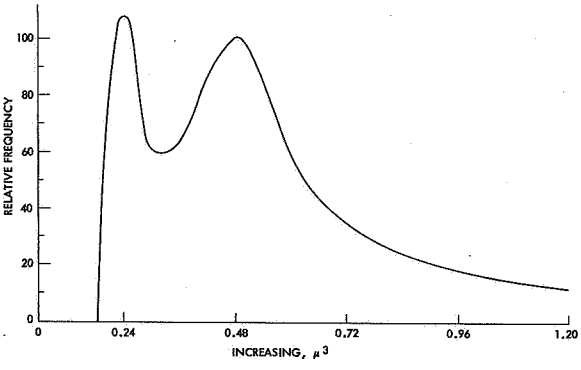

FIGURE 6.—Bacteria size distribution (increasing).

7.3 hours, oscillations were predicted (fig. 7). Although the predicted period was longer than that found experimentally (fig. 8). The predicted maxima and minima were not far from

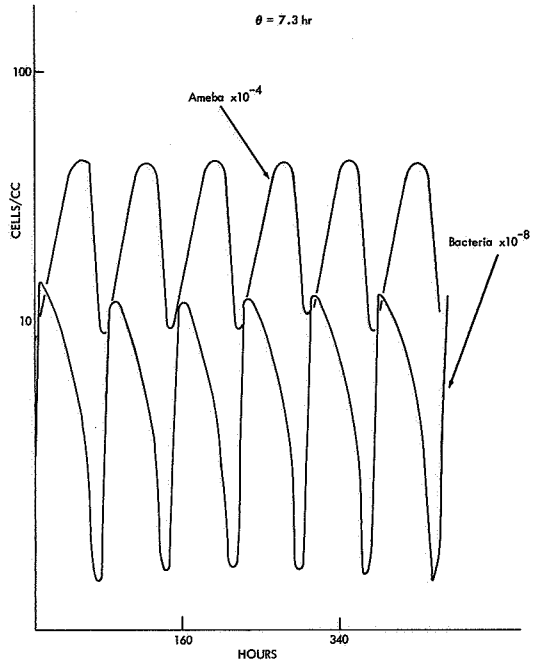

FIGURE 7.—Predicted ameba and bacterial density vs time.

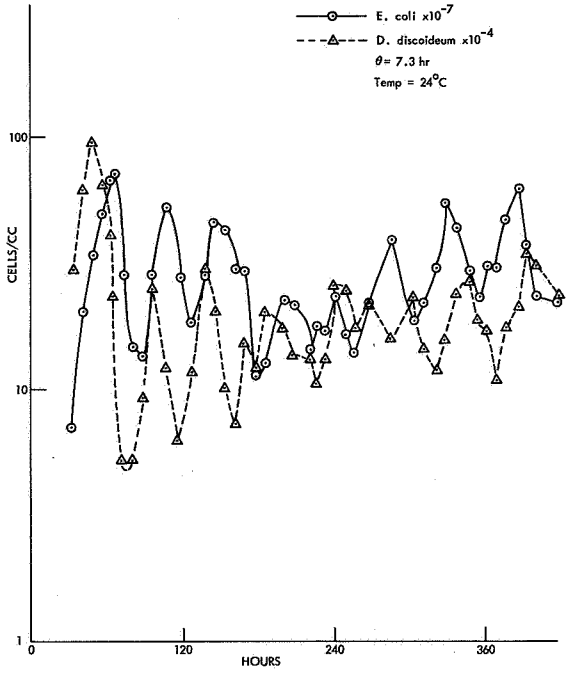

FIGURE 8.—Ameba and bacterial density vs time, at 24°C, Θ=7.3 hr.

those found experimentally. With the constants used, the predicted ameba maxima was 4.5×10^5 per ml as compared with 7.0×10^5 per ml found experimentally. With the constants used, the model predicted bacterial maxima of 14×10^8 per ml as compared with the experimental value of 13×10^8 per ml. We cannot account for the non-attainment of an apparent pseudo-steady state at this θ.

In batch culture, the model predicted the general trends that were observed experimentally, except that it predicted no lag for the bacteria (figs. 9 and 10). This shortcoming is inherent in a model that does not allow for changing bacteria structure. The experimental value for maximal ameba density of 6.6×10^5 cells per ml compares favorably with the predicted value of 6.0×10^5. The experimental value for maximal bacterial density of 14×10^8 per ml also compares favorably with the predicted value of 16×10^8 per ml.

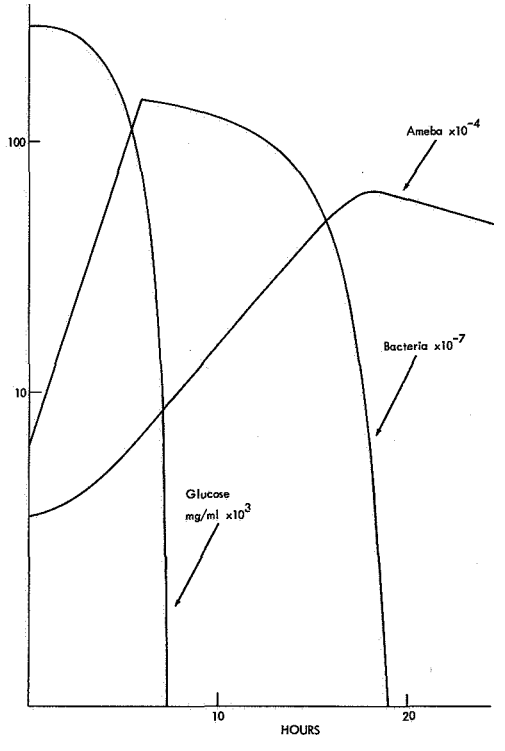

FIGURE 9.—Predicted bacterial density and glucose concentration vs time.

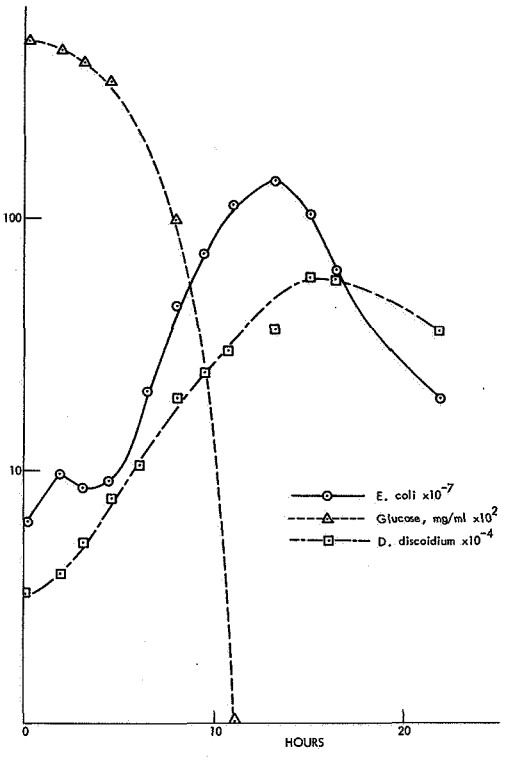

FIGURE 10.—Ameba and bacterial and glucose concentration vs time.

The reasonably good comparison of maximal bacterial and ameba numbers is encouraging; the stoichiometric constants seem fairly adequate. On the other hand, the μ's and K's for the ameba (estimated from batch experiments) leave something to be desired; μ_A actually varied from 0.24 to 0.15 hr^{-1} in batch cultures of *D. discoideum* growing on *E. coli* in medium without glucose. If the latter value had been used, the predicted period of oscillation (fig. 7) would have been closer to the observed value.

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COMMENTS

Dr. DECICCO. Did you ever limit this substance, as I presume you have to do?

Dr. TSUCHIYA. Yes. We operated at 0.5-percent glucose, which was limiting. The complete computation of the medium and so forth will be in the hand-out which Dr. Saunders will pass out later.

Dr. DECICCO. How would your picture change if you had an unlimited glucose supply?

Dr. TSUCHIYA. You would begin to introduce complications, because there is always the possibility of your getting something that may be toxic. I do not know, in the light of what Dr. Tischer had to say, whether we would encounter this. I am pretty confident, having worked with fermentations, that if you put in a great many nutrients, you will get inhibition. As a matter of fact, in the case of simple, alcohol fermentation, we have only about 20 to 30 percent. We cannot increase that, because the solution gets too toxic.

Dr. DECICCO. Let us say you doubled that to 1 percent, where presumably you are not limiting your growth any more.

Dr. TSUCHIYA. This is going to depend on the *Hydrogenomonas*.

Dr. DECICCO. I do not see that this will drastically change your picture. You expect it would, if you had an an optimal glucose supply.

Dr. TSUCHIYA. Yes. We do not want any oscillations because, after all, the bacteria are only a substrate for the amoeba. We do not want any oscillations at all in the capsule, in the lunar base, or anywhere else. For the amoeba and the bacteria, there is a definite overshoot.

Dr. DECICCO. You would still have steady state?

Dr. TSUCHIYA. Eventually, under some conditions.

Dr. DECICCO. This is why I did not understand before; I got the impression you obtained a steady state, too.

Dr. TSUCHIYA. No; I am sorry if I gave you that impression. We would not get a steady state. These chemostats were always started out with *E. coli* in the medium. We let them go for about a week until we were sure the *E. coli* were at steady state. Then we inoculated the medium with the amoeba.

Dr. COOKE. Would it not be easy to upset your steady state?

Dr. TSUCHIYA. The emphasis has been upon power and weight, but it seems to me that reliability is what really needs to be looked at. That is why Dr. Frederickson was talking about transients. We hope to be able to look at these sorts of things.

Dr. REPASKE. Is there a long delay in achieving steady state because you used a small inoculum of amoeba and were just establishing steady-state conditions, or is this fluctuation that lasts several days a usual occurrence?

Dr. TSUCHIYA. No. We actually went in with the bacteria, always operating it about 10^8 in this medium. The amoeba operated about 10^5. Our inoculum was always above these points, so we actually had to come down. That is the reason why we always start out with a very large inoculum, when we begin our chemostats.

Dr. DECICCO. Why did it take so long—360 hours in some cases—to establish a steady state?

Dr. TSUCHIYA. I am afraid of these continuous experiments which are carried out for only 1 or 2 weeks. We carried them out for months, literally. The data were not shown, because we would have had to express the time scale even more, but we have such data.

Dr. BONGERS. Do you feel that the nature of the system is such that it must come to a steady state eventu-

ally? This seems to be what the group here is thinking about, that this necessarily has to come to a steady state in the chemostat, but could not at least under some conditions. This is a continuous oscillation that continues on forever.

Dr. TSUCHIYA. This is what we found at holding time for 7.3 hours. As a matter of fact, at that point we terminated because the system kept on oscillating.

Dr. DECICCO. This is found in other predator-prey studies in all sorts of animals, that under some conditions this kind of oscillation can keep on forever.

Dr. SCHWARTZ. As far as I can see, this inoculating system is a steady state, if you take the right time scale.

Dr. TSUCHIYA. If you sample properly, and that is the reason we are trying to obtain the samples every 8 hours.

Dr. TISCHER. You had a term up here in which the numerator was about five or six things multiplied together. Was it your decision—is this a development of your own, to have this multiplicity of the numerator?

Dr. TSUCHIYA. No. The growth terms have already been suggested by Monod. They have been used by Herbert, and we have used them, too. What they have not taken into consideration—and what *we have* taken into consideration—is death.

Dr. TISCHER. I agree that bacteria are not immortal; but I wonder how hard it would be to prove this.

Dr. TSUCHIYA. Our death rates were obtained batch-culture-wise. We followed the populations; three did die out.

Dr. TISCHER. There are those, of course, who say that bacteria do not age. Obviously they die, and in order to die (one presumes) they must age, so to me this represents something of a dilemma.

Dr. TSUCHIYA. Our idea is not to talk in terms of age, because that is something very difficult to measure. You can measure in terms of the clock hour. But, as biologists, we are really interested in physiologic states.

Dr. TISCHER. But in terms of a molecular biologic interpretation of age it is rather complicated. Last year in Columbus, you mentioned a group of methods of design and analysis, called Evop's designs. Do these things represent anything in that line at all? You mentioned turbidity or growth rate, or something like that, as a result of any number of other independent factors.

Dr. FREDRICKSON. We have done this for algal growth.

Dr. TISCHER. With regard to your expressed diagrams, have you ever analyzed this for consistency or constancy of phase-angle differences? You mentioned that these curves were out-of-phase. That would mean that the phase angles might conceivably have a more or less constant relationship.

Dr. TSUCHIYA. I should think they would. That was the reason I showed one figure where we took only a portion of the data and showed a very definite relationship between glucose, *E. coli*, and *D. discoideum*.

Dr. TISCHER. I would be interested in a computer analysis of the phase-angle situation, because that would be one of the simplest things to understand. We have had one or two references here to the differences in response between pure cultures, so-called, and contaminated ones. I suggest that the group think about this situation; contamination of responses die, let us say, to contaminated cultures, is an area which there is much thinking but very little speaking and even less writing.

Dr. TSUCHIYA. We are thinking in terms of contamination, but you have to define what that means. There are mutualisms and antagonisms of all kinds; you cannot talk in terms of contamination alone. You must think in terms of contamination *type*, as Dr. Goldner was doing yesterday.

The Physiology and Biochemistry of Algae, with Special Reference to Continuous-Culture Techniques for *Chlorella*

ROBERT W. KRAUSS
University of Maryland
College Park, Maryland

To cover the physiology and biochemistry of algae in a brief statement is an awesome assignment. However, there is merit in examining briefly some of those aspects of algal physiology that are especially pertinent to the employment of these organisms in continuous-culture devices, which may be prototypes of those adaptable to life-support systems for men in space. This paper is concerned with certain features of algal growth and metabolism which must be kept in mind to ensure that cultures of microorganisms may become stable and reliable components of long-term life-support systems in closed ecological environments. Although most of these comments will be directed to photoautotrophic organisms, many of the principles pertain to heterotrophic growth as well.

CHARACTERISTICS OF GROWTH

Chlorella differs from the growth of bacteria and most unicellular fungi. The protoplast is enclosed within a wall; and although sexual reproduction is not known in *Chlorella*, autospores are formed within that cell wall. For a time, from 2 to 128 daughter cells remain enclosed within the mother cell wall. When the cell wall is ruptured by a combination of pressure and enzymatic action, it generally hydrolyzes. Because the wall is made primarily of a cellulose polysaccharide, the products of hydrolysis are reabsorbed by the algae.

During the maturation of the small daughter cells of *Chlorella*, numerous changes in metabolic activity, as well as in chemical composition, take place. The various phenomena that occur during the growth and development of the algal cell from its nascent condition to maturity and to the stage of reproduction have been studied and characterized (refs. 1–3). This is quite different from the situation in the bacterial life cycle, where the organism is split into two nearly equal parts with a much less conspicuous change in biochemical characteristics as each segment grows to reproductive size again.

Normally, algal cultures show a random distribution between the young and old cells and the intermediate stages. However, under certain cultural regimes, the population may be skewed to either greater or lesser numbers of any of the intermediate stages of the cell. The random nature of a normal cell population, as well as the salient features in the morphology of the cells of *Chlorella*, are given in figure 1. This figure shows spherical, green cells with net-like chloroplasts and illustrates a range in cell size from the young to adult reproducing cells. It should be remembered that this is only one of some 40 known species of *Chlorella*, which vary dramatically in morphology, pigment concentration, temperature tolerances, nutritional requirements, and numerous biochemical characteristics. It should also be recalled that a culture's appearance can be dramatically altered by environmental changes; different species react quite differently to various sets of environmental characteristics. An illustrative example of the range encountered in the genus is the appearance of cells of *Chlorella protothecoides* Krüger grown in darkness. These colorless cells (fig. 2) are devoid of chloroplasts and contain an entirely different spectrum of organic components.

In observing and recording the performance of a *Chlorella* culture to extrapolate possible performance in life-support systems, scientists may report gas exchange in terms of either carbon-dioxide absorption or oxygen evolution, as measured by electrodes, in Warburg flasks

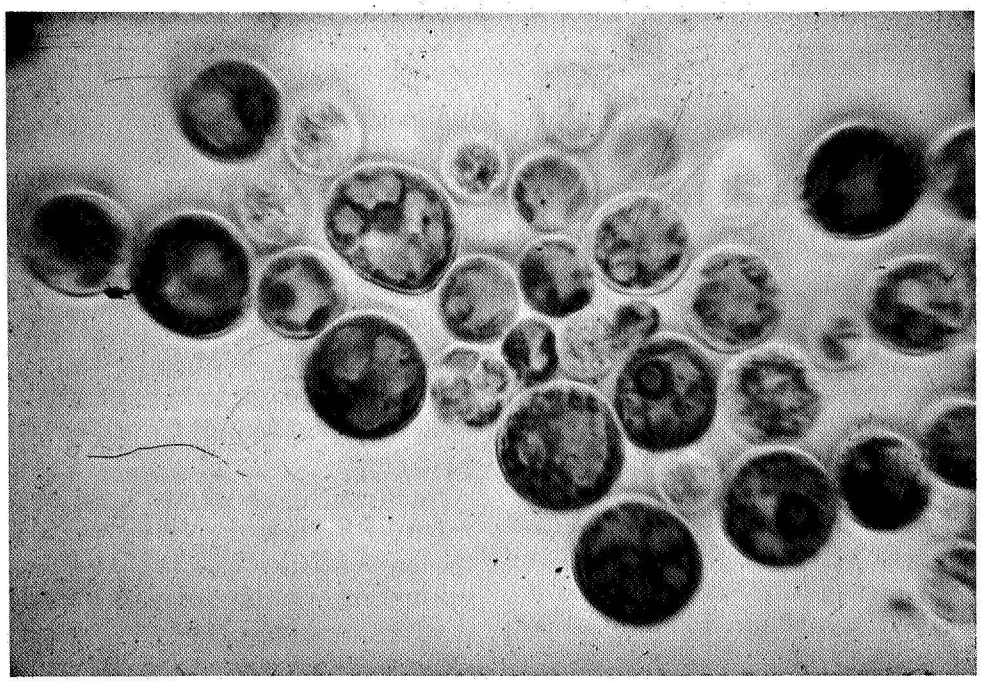

FIGURE 1.—Chlorella emersonii Shihira and Krauss grown photographically in inorganic media in the light.

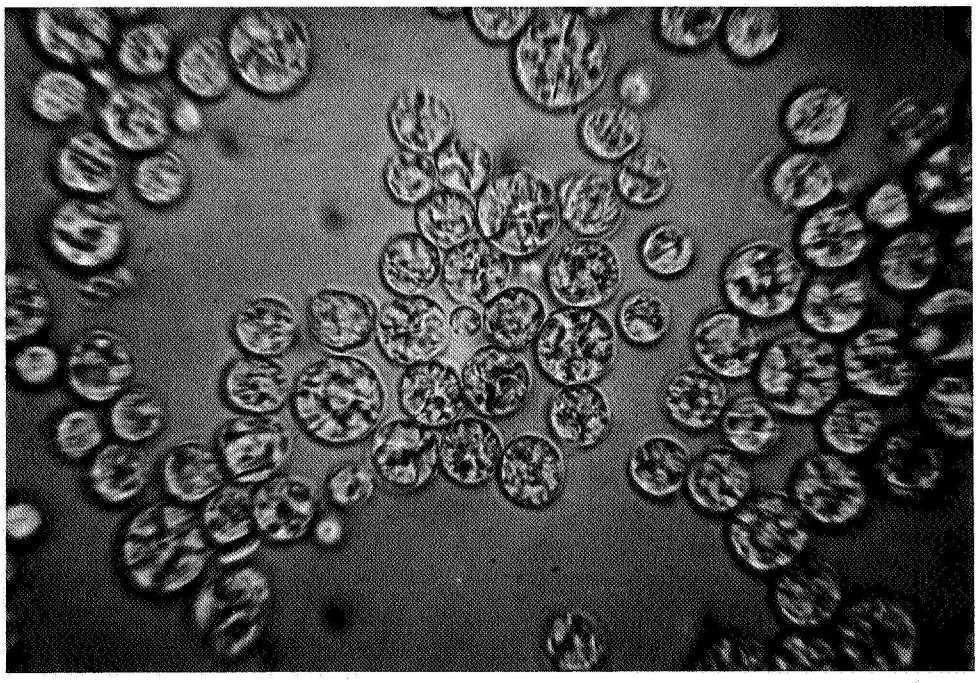

FIGURE 2.—Chlorella protothecoides Krüger grown heterothrophically in the dark on glucose.

and in other devices. Cell production is measured in terms of optical density, cell number, dry weight, or cell volume. Reasonable accuracy in translating growth data into gas exchange data can be attained if one knows the chemical composition of the cellular product. Consequently, we have focused our attention on the growth of the algae as a measure of gas production, simply because it is necessary to produce algal cells that act essentially as oxidants for water and, in turn, permit the evolution of oxygen.

There are several features of algal growth that should be emphasized. Figure 3 shows a normal sigmoid growth curve that is typical of any organism grown in a batch culture where some limiting factor intrudes—whether it be light, secretion of an autoinhibitor by the organism, or the depletion of the nutrient source. Also plotted is the curve showing the shift in growth rate in a culture demonstrating sigmoid growth. This curve is high and flat during the exponential stage of growth and then drops progressively to the point where growth is stationary. Continuous-culture devices are constructed to block the reduction in growth by maintaining a constant population density at some preselected point. If the objective is maximum yield, the point is selected where the product of growth rate times population gives a maximum figure. Of course, the population that gives a maximum yield, and the growth rate sustaining that yield, will differ dramatically with the geometry of the culture vessel and with the composition of the nutrient solution.

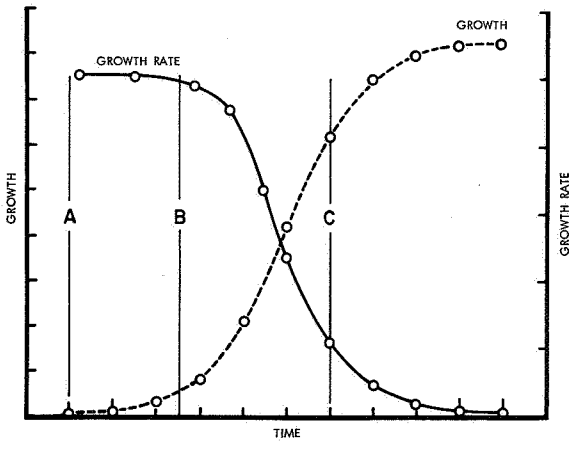

FIGURE 3.—The relation between the plots of a typical sigmoid growth curve of an algal culture and the growth rate, K calculated for that same culture using the formula, $\log_2 \frac{C_2}{C_1} = K\ (t_2 - t_1)$.

There are two formulas commonly used in computing growth rate (table I). The first is the familiar equation for microbial growth in which rate is reported in terms of logarithms to the base 2 (often discussed as doublings per day). Although the second formula (ref. 4) has often been overlooked, it is used in calculating rate from dilution data in a chemostat type of culture apparatus. This formula must be used where cell population and culture volume are constant. It should be remembered that, in such situations, the growth rate does not reveal the yield. For example, if the growth rate is 2 (2 doublings per day), the yield will be much less. This is true simply because the increment of growth must be removed as soon as that increment is produced. We are, therefore, dealing with an arithmetical removal from what we customarily regard as growth measured by an exponential constant. For this reason, extrapolations based on maximally observed laboratory growth rates cannot be taken directly as an indication of organism productivity in an apparatus of constant volume, regardless of design excellence.

TABLE I.—*Growth Rate Equations*

General
$\log_2 \frac{C_2}{C_1} = K\ (t_2 - t_1)$
$\log_2 = \log_{10} \times 3.32$
C_1 = concentration (dry wt., cell no., o.d., etc.) at beginning of the time period, t_1
C_2 = concentration (dry wt., cell no., o.d., etc.) at end of the time period, t_2
K = growth rate, doublings per day
$t_2 - t_1$ = 24 hr usually taken as 1
Recyclostat or constant-volume chemostats
$\phi = VK\ (0.69)$
ϕ = liters added per 24 hr
V = liters of constant culture volume
K = growth rate, doublings per day

The potential performance of a given species of algae can, of course, be predicted from laboratory determination of growth rate in batch culture. For instance, the comparative growth rates and optimal physical characteristics and requirements for two species of *Chlorella* can be seen in table II. The growth rate of 10 species accurately reflects the superior performance of *Chlorella sorokiniana* Shihira and Krauss at its optimal temperature of 39° C. The rates of photosynthesis and glucose respiration are also accurately reflected. However, the optimal performance characteristics must be viewed with caution when extrapolating performance in constant population culture devices. In order to obtain optimal yields, these will be operating at less than the maximum growth rate, and cells will be exposed to light intensities that may be much more or much less than in the dilute populations employed when determining

TABLE II.—*Characteristics of Growth, Photosynthesis, and Respiration for High and Low Temperature Strains of* Chlorella Pyrenoidosa

Characteristic	Strain of *Chlorella*	
	Emerson	7-11-05
Temperature (°C) optimum for:		
growth	25–26	38–39
photosynthesis	32–35	40–42
endogenous respiration	30	40–42
glucose respiration	30	40–42
Growth rate at light saturation (number of doublings per day):		
at 25°C	3. 1	3. 0
at 39°C	----------	9. 2
Rate of apparent photosynthesis at light saturation (mm^3O_2/mm^3 cells/hr):		
at 25°C	43	47
at 39°C	rapidly declining	170
Rate of glucose respiration (mm^3O_2/mm^3 cells/hr):		
at 25°C	4. 5	8
at 39°C	1. 6	18
Saturating light intensity for growth (ft-c):		
at 25°C	500	500
at 39°C	----------	1400

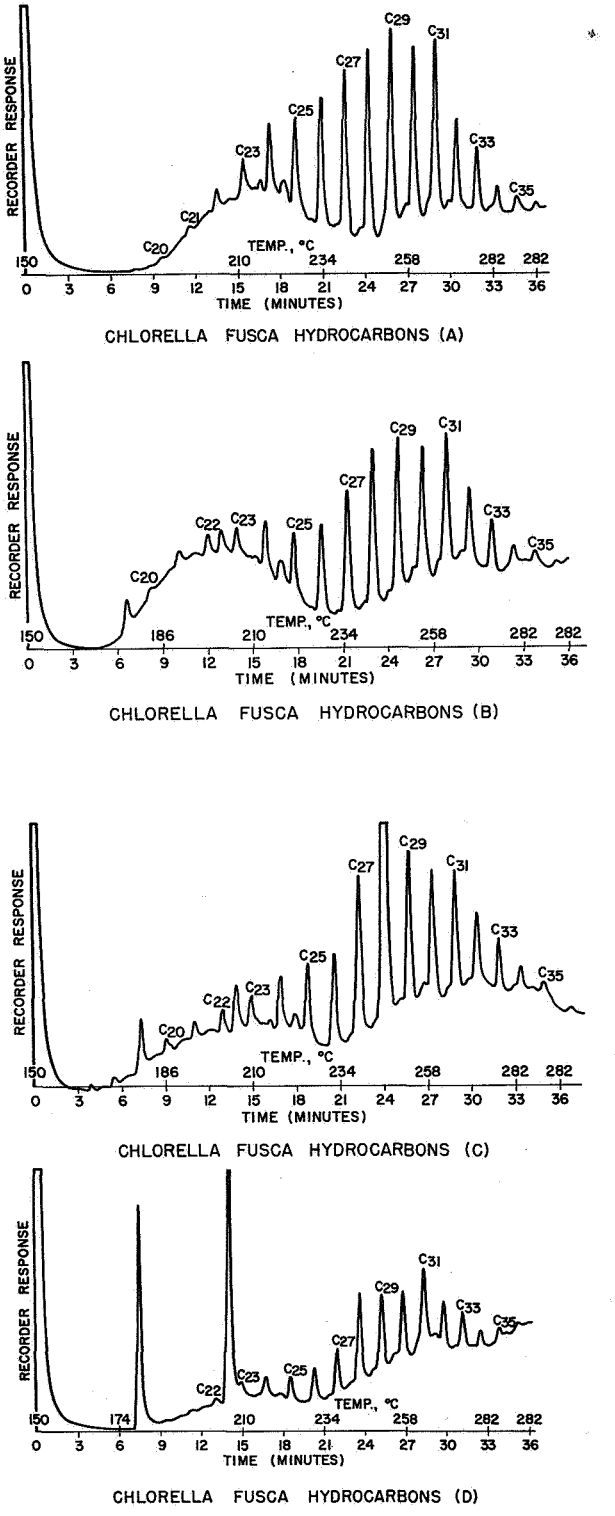

FIGURE 4.—The hydrocarbons found in Chlorella fusca Shihira and Krauss as the culture density increases from an optical density of 0.3 to a maximum of 4.7.

optimal performance. Light, especially, becomes limiting at high populations regardless of the intensity of the source.

A good example of how cellular characteristics change when light intensity changes is given in table III. As light intensity increases, the chlorophyll content of the cells drops. This is a phenomenon that has been well-known for some time. However, not only does the chlorophyll content change, but the ratio of chlorophyll *a* to *b* also shifts. Such changes in the photosynthetic apparatus also modify the performance characteristics of the organism just as much as if an entirely different species were to be introduced into the culture apparatus. In other words, performance parameters must be determined under a rigidly specified set of culture parameters.

Variations in the organic components of a given species of *Chlorella* can also be matched by variations in the composition of the potential food products produced by different species of algae. Figure 4 indicates the changes that can take place in the hydrocarbon composition of *Chlorella fusca* Shihira and Krauss as density and culture age progress. It is obvious that there is a dramatic increase in the concentration of the shorter-chain hydrocarbons as the culture ages. The growing evidence of the magnitude of such shifts in the biochemical components

TABLE III.—*The Relationship of Light Intensity to Dry Weight, Total Chlorophyll, and Chlorophyll* a *and* b *in Cells of* Chlorella Vannielii

Light intensity	Correct o.d. 550 mμ	Dry weight mg/50 ml	mg chl/g dry weight				
			Chl *a*	Chl *b*	*a* + *b*	*a*/*b*	Average
100	0. 6	13. 5	32. 83	12. 34	45. 17	2. 66	2. 88
100	0. 6	14. 0	36. 04	12. 06	48. 10	2. 99	
300	0. 6	17. 0	29. 07	9. 87	38. 94	2. 94	2. 96
300	0. 6	19. 0	21. 87	7. 30	29. 17	2. 99	
900	0. 6	23. 0	12. 13	2. 95	15. 08	4. 11	4. 04
900	0. 6	24. 0	11. 52	2. 90	14. 42	3. 97	
2700	0. 6	20. 0	7. 66	1. 57	9. 23	4. 88	4. 84
2700	0. 6	22. 0	8. 83	1. 84	10. 67	4. 80	
100	1. 2	29. 5	25. 11	8. 06	33. 17	3. 12	2. 95
100	1. 2	29. 0	34. 58	12. 39	46. 97	2. 79	
300	1. 2	36. 0	23. 38	7. 82	31. 20	2. 99	2. 97
300	1. 2	35. 0	23. 90	8. 13	32. 03	2. 94	
900	1. 2	39. 0	15. 60	4. 23	19. 83	3. 69	3. 49
900	1. 2	37. 5	15. 08	4. 21	19. 29	3. 58	
2700	1. 2	40. 0	9. 72	1. 86	11. 58	5. 22	5. 15
2700	1. 2	43. 0	10. 00	1. 97	11. 97	5. 08	

of *Chlorella* dictates caution in extrapolating not only cell growth rates in various culture situations, but also the organic composition of those cells.

As a final illustration of the problem in predicting cell performance, it is useful to consider the recent experiments of Epel and Krauss (ref. 5). These studies, based on an earlier observation that cell division was inhibited by high light intensities (ref. 6), used the colorless counterpart of *Chlorella vannielli* Shihira and Krauss, which is *Prototheca zopfii* Krüger. The performance of *Prototheca* under various light intensities is given in figure 5. These data show that the growth rate of this organism was greatly inhibited by increasing light intensities, even though it is not a photosynthetic organism. The culmination of a series of studies indicated that the inhibition in the growth was manifest in a delay in cell division. The action spectrum given in figure 6 indicates that there

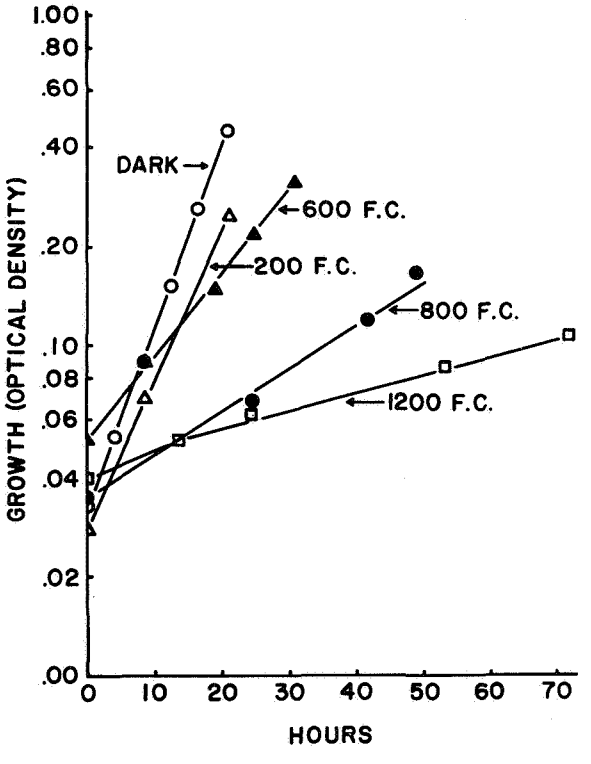

FIGURE 5.—A set of typical growth curves for Prototheca zopfii Krüger grown in the dark, and at illuminances of 200, 600, and 1200 foot-candles from General Electric cool-white fluorescent lamps.

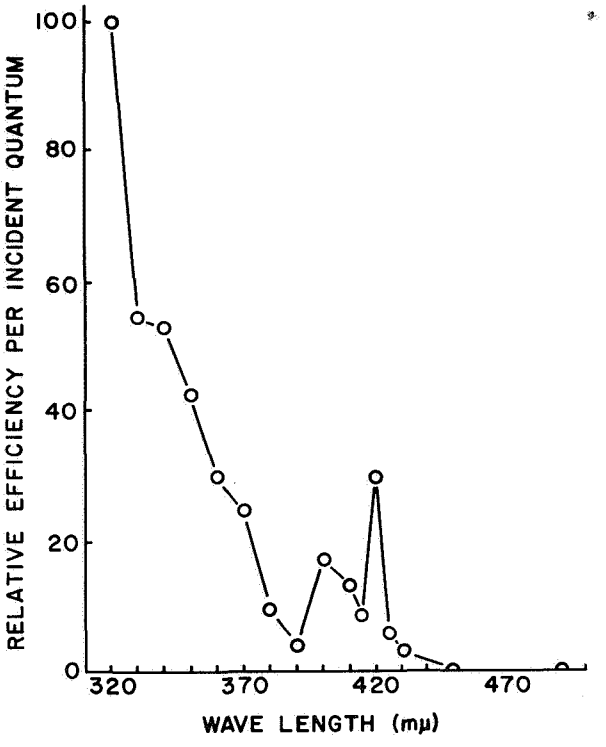

FIGURE 6.—The action spectrum for inhibition of growth in Prototheca zopfii adjusted to equal quantum intensity, and normalized to 100 at 3200A°. The intensity employed at 3200A° was 4×10^{14} quanta/cm^2 sec., and the irradiation period was 12 hours. For this intensity and time of irradition, the percent inhibition at 3200A° was 37 percent.

is an inhibitory peak at 420 mμ and that this inhibition in the blue can be obtained regardless of the light source. Additional evidence leads us to suspect that a cytochrome pigment is responsible for this damage to cell growth. Nevertheless, it is apparent that, in considering photosynthesis as a mechanism for producing oxygen and synthesizing food, one is forced to deal with competing photochemical processes. Although difficult to determine, the optimal balance provides the opportunity to obtain a maximal and, perhaps unpredictably high, performance from the alga.

THE RECYCLOSTAT

Associated with studies of algal metabolism have been experiments to explore algal growth in typical culture devices. These devices might

be considered prototypes of the kinds of apparatus needed in closed ecological systems in space—either for long-term space flight or for moon bases. The requirements for an algal or photosynthetic microorganismal culture system suitable for space flight include: (1) provision for illumination, (2) a method of CO_2 circulation and oxygen removal, (3) a mechanism for harvesting of the algae, (4) a recycling water-use system, (5) temperature controls, (6) protection from contamination, (7) introduction of inorganic nutrients, and (8) automatic controls and recorders for continuous long-term operations.

The device developed in our laboratory for studying continuous, automatically recycled algae cultures has been named the "Recyclostat" (fig. 7). The wiring diagram is shown in figure 8, and two alternative culture chambers housing the algae in this apparatus are shown in figures 9 and 10. In brief, the device operates as follows: The algal culture is maintained in a glass chamber illuminated by incandescent or fluorescent lights mounted within the culture apparatus or maintained and cooled on the outside. A glass cooling coil at the center of the culture controls the temperature. A 1-percent carbon dioxide-in-air mixture is bubbled through

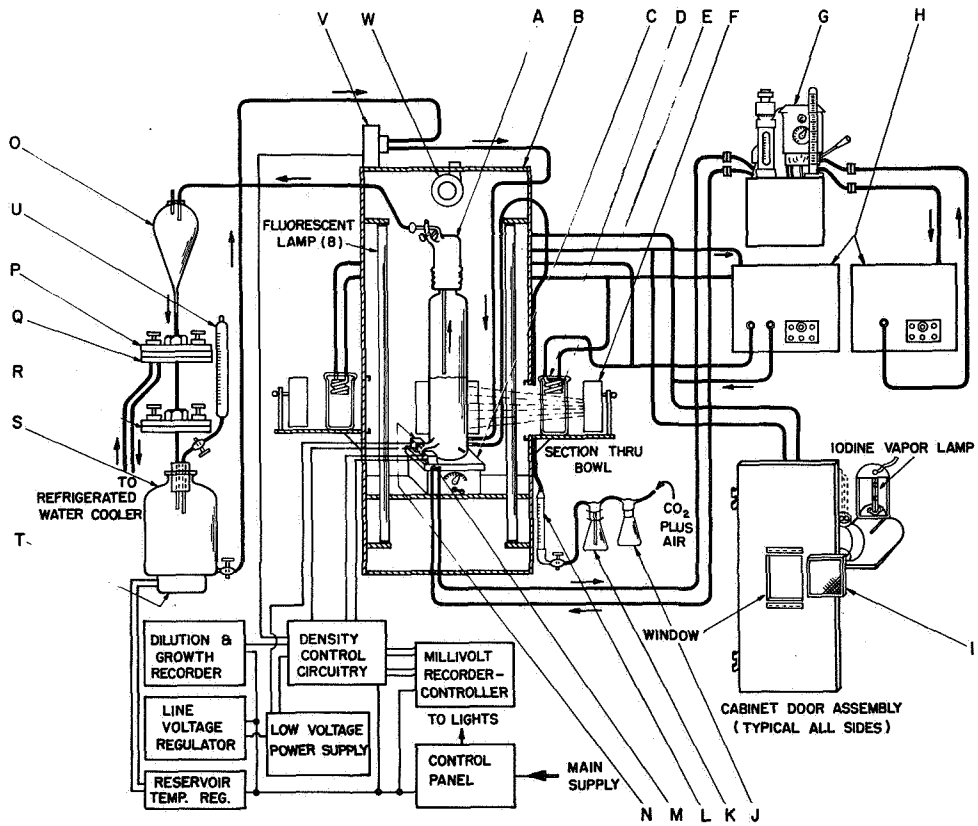

FIGURE 7.—The Recyclostat—a continuous-culture apparatus for algal experiments in sterile recycled media under controlled light and temperatures. The system provides for sterile culture, harvest, and medium recycling that is continuous and automatic. A—culture chamber (including top and bottom); B—cabinet; C—electric stirrer; D—water filter; E—cooling coil; F—high intensity "Quartzline" iodine vapor lamp; G—temperature regulator; H—potentiometer; J—water cooler; K—gas filter (cotton); L—gas filter (water); M—flowmeter; N—photo duo diode; O—reference lamp (V_2); P—vacuum pump; Q—timer; R—stopcocks; S—recycled medium reservoir; T—solenoid (L_1); U—vacuum trap; V—intermediate supernatant reservoir; W—membrane bacterial filter; X—overflow receiver; Y—flow inductor; Z—fluorescent lamp.

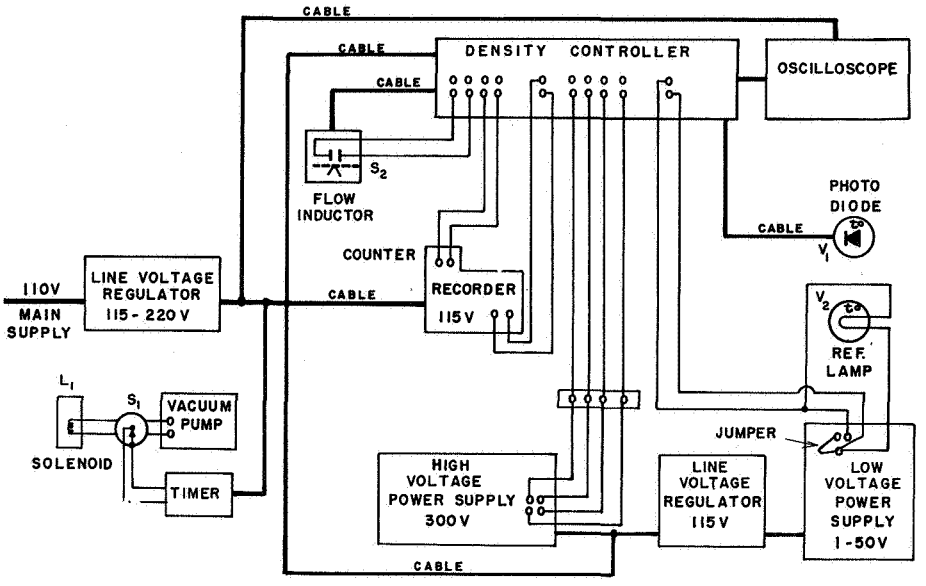

FIGURE 8.—Recyclostat wiring diagram.

the culture medium. The optical density is recorded by photodiodes mounted on either side of a small bulge at the bottom of the chamber. The culture is agitated by a magnetic stirrer. When the population density reaches a preset level, the photodiode signals for the introduction of an aliquot of culture medium, which is pumped through a peristaltic pump from a pasteurized reservoir. When the appropriate amount of fresh medium has been introduced to dilute the culture to its preset level, the equivalent amount of culture is forced out of the exhaust port by the CO_2-in-air pressure maintained within the culture chamber. The effluent cells and media pass through an air trap and are filtered on a bacteriological millipore filter. The medium passes through another microbiological filter (which serves as a port for removal of the harvested cells) and back into the reservoir from which it is recycled to the culture.

The culture medium maintained in the reservoir is given in table IV. Each day a small aliquot of a concentrated culture medium is introduced to compensate for the inorganic nutrients (determined spectrographically) that are to be removed by the algal cells produced during that day. The number of algal cells produced can be measured by a readout apparatus mounted next to the chamber. The formula for the concentrated medium, which is strongly acid and consists primarily of nitric acid, is given in table V. The entire system can be operated free of bacteria and has been operated in this fashion for periods of up to 6 weeks. Suitable bacteriological traps, including filters,

TABLE IV.—*Inorganic Medium for the Autotrophic Culture of* Chlorella Vannielii *Shihira and Krauss*

Salts	Grams per liter of distilled water
KNO_3	1.00
$MgSO_4 \cdot 7H_2O$	0.25
KH_2PO_4	1.00
EDTA·NaFe[a]	0.0385
EDTA·Na_2Mn[a]	0.0071
EDTA·Na_2Ca[a]	0.0077
EDTA·NaCo[a]	0.0093
EDTA·Na_2Cu[a]	0.0077
EDTA·Na_2Zn[a]	0.0067
H_3BO_3[a]	0.0057
MoO_3[a]	0.0015

[a] Individual stock solution of each of the trace element compounds are maintained as the sources for each batch of medium.

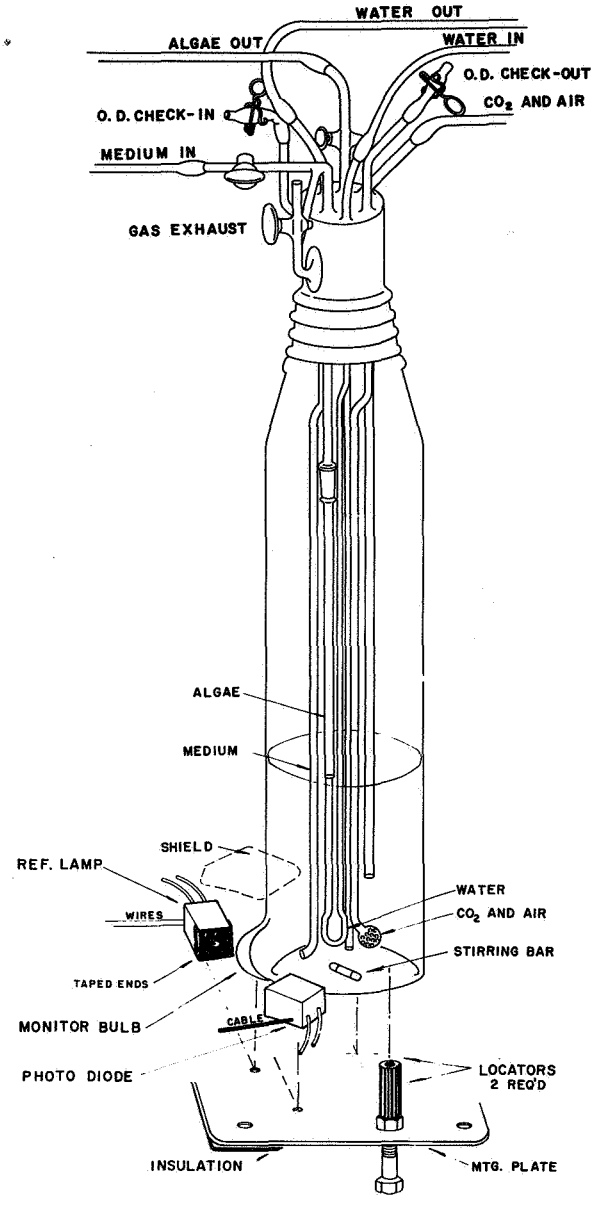

FIGURE 9.—Recyclostat assembly for the continuous recycling of Chlorella cultures.

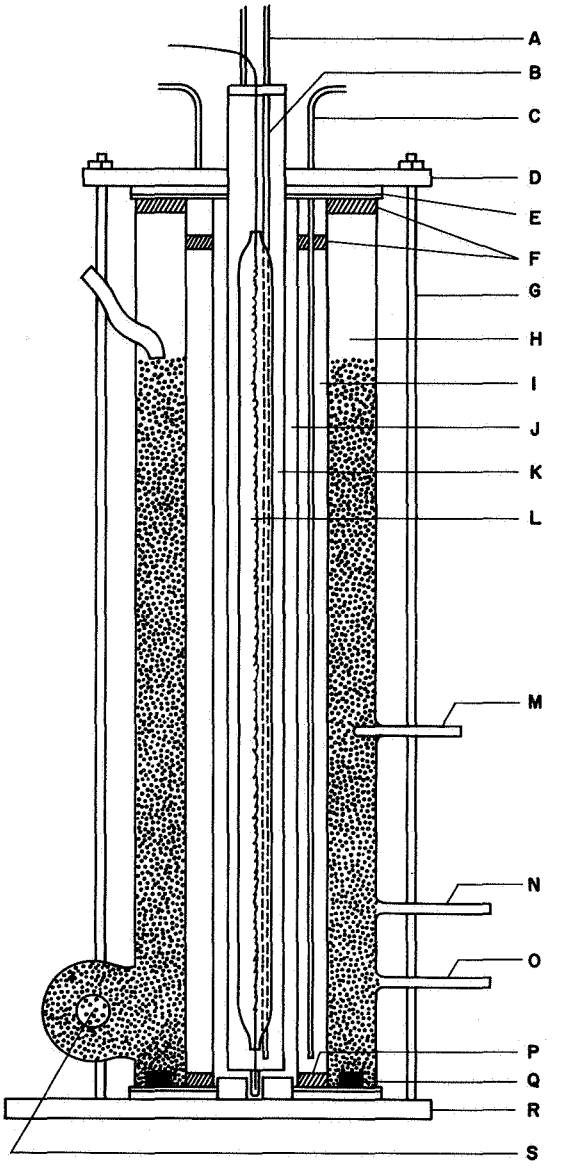

FIGURE 10.—The culture chamber of the Recyclostat. A—metallic water inlet for lamp cooling jacket; B—glass water director tube for lamp cooling jacket; C—metallic coolant inlet for culture-temperature control jacket; D—end-plate, chamber assembly clamp; E—gasket assembly, 3/16-inch hard rubber and 0.030-inch Teflon sheet; F—spacers; G—bolt, chamber assembly clamp; H—culture jacket; I—coolant jacket, culture temperature control; J—air space; K—water jacket, lamp cooling; L—lamp, 1500-W incandescent (quartz); M—well, temperature-control probe; N—medium inlet; O—gas inlet; P—spacers; Q—magnetic-disc stirrers; R—end-plate, chamber assembly clamp; S—well, density controller optics.

pasteurizers, and air traps, have been introduced at various parts of the apparatus.

Considerable data have been obtained which show consistent long-term yields for days or weeks in the Recyclostat. The developing pattern is that which could be predicted from the theoretical considerations of growth mentioned earlier in this paper. Figure 11 is a graph of the typical response of a culture of *Chlorella van-*

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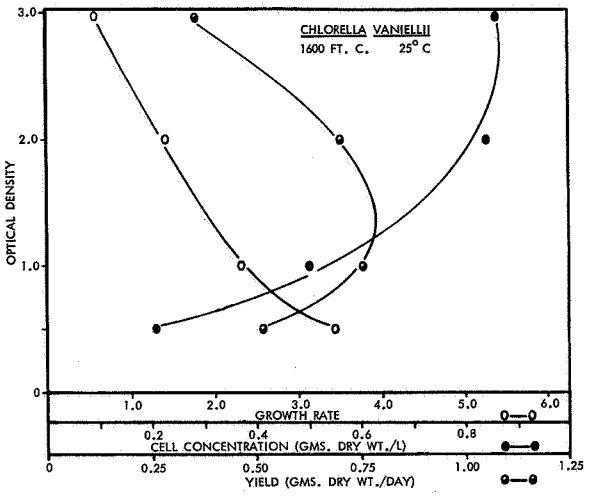

FIGURE 11.—The yield, growth rate, and dry weight per liter of culture of Chlorella vannielii Shihira and Krauss grown sterilely at 1600 f-c in the Recyclostat at 25°C.

TABLE V.—*Replacement Nutrient Formula for Culture of* Chlorella *in the Recyclostat*

Salts	Grams per liter of distilled water
HNO_3	91. 4
KH_2PO	0. 176
$MgSO_4$	0. 90
MgO	0. 006
EDTA·Fe	0. 003
EDTA·Mn	0. 0003
EDTA·Ca	0. 0016
EDTA·Cu	0. 00013
EDTA·Zn	0. 00008
EDTA·Co	0. 00001

nielli to different cell populations at a high intensity of 1600 foot-candles. Growth rate, cell concentration, and the yield are plotted in this graph. It can be seen that maximum yield is obtained at a growth rate well below the optimum at a cell concentration of approximately 0.6 g dry weight per liter. If we examine a similar experiment run with the well-known, high-temperature strain *Chlorella sorokiniana* at this light intensity, the maximum yield is less (fig. 12). However, when *Chlorella sorokiniana* is grown at 7800 foot-candles in quite dense solu-

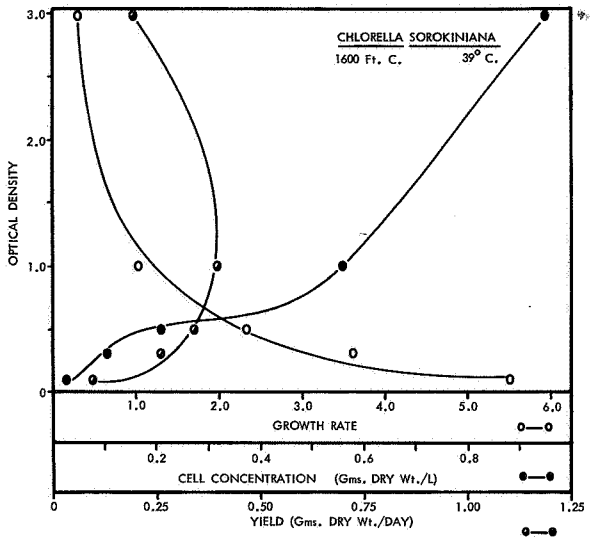

FIGURE 12.—The yield, growth rate, and dry weight per liter of culture of Chlorella sorokiniana Shihira and Krauss grown sterilely at 1600 f-c in the Recyclostat at 39° C.

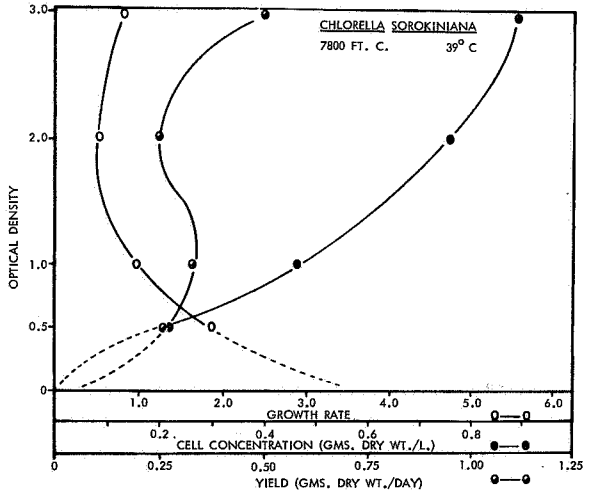

FIGURE 13.—The yield, growth rate, and dry weight per liter of culture of Chlorella sorokiniana Shihira and Krauss grown sterilely at 7800 f-c in the Recyclostat at 39°C.

tions, there is a change in the direction of the curve, indicating a resurgence in yield after a cell concentration of 0.4g per liter has been reached (fig. 13). These data indicated that, although *Chlorella sorokiniana* does not out-yield

TABLE VI.—*Comparison of Rates and Yields of* Chlorella Vannielii *in the Recyclostat and in Test Tubes as Related to Human O_2 Requirements*

Parameters	Recyclostat equivalents (8-cm diameter)	Test tube equivalents (2-cm diameter)
Algal growth rate doublings/day	2.7	3.3
Optical density	1.0	1.5
Maximum algal yield grams dry wt./liter/day	1.0	1.5
O_2 liters/gram algae	1.4	1.4
O_2 requirement liters/man/day	610.0	610.0
Algal volume required for O_2 one man/day	436.0	291.0
Surface area—cm^2/liter	440.0	1760.0
Algal illuminated surface me/man/day	19.3	51.2

the more typical strains of *Chlorella* under normal conditions, the growth begins to increase at higher intensities and higher cell concentrations. In order to continue an examination of the performance of *Chlorella sorokiniana* at high cell densities and high light intensities, an alternate chamber (fig. 13) is now in use. In this chamber a much greater surface per unit volume can be illuminated, and we will soon be able to report in some detail the long-term performance data of this species at extreme intensities and culture densities.

CONCLUSIONS

Although many conclusions can be drawn from the various studies made during recent years, it seems correct to say that the optimal performance characteristics of *Chlorella* in the Recyclostat are as given in table VI. The optimal long-term cell production has been not more than 1 gram per liter per day. In order to supply the human oxygen requirement a volume of 436 liters per man is required. However, these experiments were run primarily to gain information on long-term performance rather than to establish the maximum efficiency of energy utilization or the configurations necessary for maximum production. The second column in table VI indicates that considerable gains in culture volume efficiency can be obtained by increasing the amount of surface illuminated per unit volume of algae. Any increase in the surface volume ratio would result in a reduction of the needed volume accompanied by a concomitant increase in yield and efficiency. This will, at the same time, impose certain engineering difficulties. There is little doubt, from extrapolations of our own work and others, that the yield can be increased well above that which we have seen in the current design of our culture chamber. Our experience with the new chamber already indicates a major gain in efficiency. However, major problem in the coming years will be how best to trade off design against efficiency.

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COMMENTS

Dr. JENKINS. I would like to know what groups are working on continuous culture or recycling of algae.

Dr. KRAUSS. To the best of my knowledge, we are the only group who has a recycling system in operation.

Dr. JENKINS. Is there anyone else working on the chemostat or turbidostat principle of continuous culture?

Dr. KRAUSS. The turbidostat principle is a fairly old one and, in fact, turbidostats are used by Calvin's laboratory simply to give him a production of cells he can use. Dr. Myers in Texas had one of these. We have some of them in operation at our laboratory. They are not so unusual.

Dr. JENKINS. Is this on a continuous basis?

Dr. KRAUSS. Yes. The point is that in the turbidostat, chemostat, and others, you are essentially washing fresh medium through the culture all the time. We have imposed a more severe requirement on our system in being able to balance the nutrient removal in the recycling system with the replacement of just nutrients, not a lot of fresh medium.

Dr. BONGERS. Is there any relation between the amount of removal and the amount of nutrient? Do you see a much higher rate of cells if you increase organic concentrations of the medium?

Dr. KRAUSS. We have done this. The medium I showed you is one of many. You do not want the medium too concentrated because one gets into coprecipitation problems.

From the chemist's point of view, this is a complex organic medium. We like to keep the concentration reasonably dilute. Our medium always remains as a background to the culture in the recycling system, with a great deal of latitude. The culture could run on that medium for several days with no replacement, but we maintain that background by putting back the small amounts taken out by the algae each day. We could go higher.

Dr. KOK. How critical is the mineral nutrition?

Dr. KRAUSS. It depends on what you are doing. Of course, as far as inorganic nutrition is concerned, one can get optimal rates in batch cultures with all kinds of media. But in a recycling system—and I insist this is ultimately the device you will have to use in a spaceship—you cannot throw that medium out the window. You have to recycle; it becomes critical. Any error in putting in too little or too much of the inorganic nutrients will ruin your growth rate and ruin your yield.

For instance, suppose you put in just a little bit more copper each time than the cells are actually taking out. For a few weeks, this will not make any difference. copper concentration until you find yourself in trouble.

All things being equal, I would rather err on the side of a dilute nutrient medium than a concentrate.

Dr. BONGERS. Can you rely on measuring one nutrient constituent? Presume that you measure only phosphate. You know how much phosphate the cells have taken up to grow so many cells. Can we say they will also have taken up so much magnesium, so much copper, so much nickel, etc?

Dr. KRAUSS. If you achieve essentially the steady state that we are after here—and it is not easy—and you have good spectrographic analysis of those cells, there is no reason why you cannot pretty well balance it. Perhaps every 3 or 4 months you may want to start afresh just to be sure you are not getting out of line.

There is every reason to believe this can be done. With higher plants, we have used a lot of automatic dilution techniques that pretty well maintain that medium at about the right level of each of the nutrients for the organism.

Dr. BONGERS. This is more for control than anything else. Do we have to measure all those elements every time, or can you just measure one and hope the others will fall in line?

Dr. KRAUSS. We do not measure it every time. We simply have cells that are produced in the way in which cells are being produced here. We have the analysis on those, and we use this as a typical formula. Certainly, the experiments we have run with our replacement formula—which, I am sure, is not perfect—have been adequate to maintain growth for over a month, which is an indication that we are on the right track. You will have to do this with *Hydrogenomonas* too. The principles we developed here are the ones you have yet to become involved with in your turbidostat.

Dr. FREDRICKSON. As I understand, your recycle must have a buildup of products of metabolism in the medium. Presumably your filtration removes some of them, but I assume that it does not remove all of them. Therefore, over a very long period of time, you do not have a truly steady state situation. You have sort of a pseudosteady state.

If you agree, how long can you operate your system before this buildup of metabolic products does start to reduce your growth rate?

Dr. KRAUSS. I can agree with what you have said by saying yes and no. The recycle medium in algal-free form does increase the inorganic components—particularly early in the culture of the cells. I hope there will be a Ph.D. thesis out on this pretty soon. We have done analyses on fatty acids and things that build up. Interestingly enough, after a certain level of these seem to be attained, the algae seem to absorb them

most as fast as they produce them, so one approaches a fairly steady concentration.

I think you can recognize from the schematic systems diagram that there is an opportunity to get at that medium. You do not need to let it go back through each time. There are ways, if something does develop, of getting it out without too much trouble. But, up until this point, we do not feel we are in any serious trouble with autoinhibitors. Again, this depends on the organism. Some species of *Chlorella* put out nasty inhibitors, and the system would come to a grinding halt if you should use the wrong species. The species we have been using seem to have a good tolerance for what they secrete.

Dr. TSUCHIYA. Does this apply to nitrogen compounds?

Dr. KRAUSS. Yes. It appears, from our dilution data, that algae are taking in more nitrogen than they should. You can see this nicely because you have all kinds of checks and balances in this system. Using nitrate and nitric acid as the replacement product, you can get an added check to your calculations, depending on what the pH does. If the pH goes up, it means you are not adding enough nitrate to keep it at 6.5 where we maintain it. If it starts to shift upward—this takes a while, but you see the tendency to go up—you simply increase the amount of concentrated supplement that you are using, because it is very acid, and bring it back into line. At least the preliminary data indicate that there is more nitrogen going in than probably should. How much more, it is hard to say. Maybe 10 percent. What the cells are doing with it, we really do not know. Microorganisms, as you well know, do all kinds of interesting things with nitrogen. They secrete it and change it to ammonia. This can be lost from the medium in the form of ammonia gas, depending on pH and so forth. This is also a problem we have to face, and I am sure the *Hydrogenomonas* people will have to face.

Dr. TSUCHIYA. I wonder specifically about the accumulation of organic amines.

Dr. KRAUSS. Our data so far have not indicated a major accumulation of them. If one takes blue-green algae as a perfect example of what the algae do and subjects them to a very fine diet of N_2 gas and allows them to reduce the N_2 to ammonia or to nitrate, which is a very good trick, one would consider this nitrogen that they had gone to all the trouble of reducing for amino acids and so forth would be very precious. The facts are, however, that they secrete over 50 percent of what they reduce. It is just lost. This is the really critical limiting thing for the organism. Why it would do that is hard to tell, but one has to be careful in dealing with these critters. They do funny things.

Dr. SCHWARTZ. I presume the system has a way of dealing with the volatiles that come over with the gases.

Dr. KRAUSS. We have not paid much attention to any volatile compounds that come out. This may be where our nitrogen loss is occurring. We recognize the amount of work that must go into monitoring all the things we have. Thus, we simply monitor one segment at a time and hope you learn something. We have been concerned about this, however.

Dr. BYERS. Do you have much of a problem with foaming?

Dr. KRAUSS. None at all.

Dr. FREDRICKSON. You had something against optical density which showed the point of depletion. Did you offer an explanation for the increase of the yield at the high light intensity?

Dr. KRAUSS. That is what we are excited about. It is something about this shift in chlorophyll ration, I think, that is allowing this particular organism to get away with that. You must remember that these organisms are seeing almost 10 000 foot-candles right at the culture surface. It was a source of amazement to us that they would take that in the big volume cultures. They look as though they would die right away. But, giving them a little time and moving them gradually in this position so they can shift their chlorophyll situation and so forth, they will grow at fantastically high light intensity. Not well, but they grow. What you do in these dense cultures is cut down on the amount of light that a given cell sees per unit time so you can get away with a bright source and grow them in fairly dense cultures.

The Rate-Limiting Reaction in Photosynthesis

BESSEL KOK
Research Institute for Advanced Studies
Baltimore, Maryland

The subject of photosynthesis, specifically of biological gas exchangers, is important today. Aside from the question of whether photosynthesis, chemosynthesis, or either will support astronauts in space, advances in these areas will help the attainment of a wider objective, possibly one of the most important byproducts of space reseach: an increase of the world's food supply. We cannot predict whether this will be through better use of solar energy in industrialized photosynthesis or through chemosynthesis using atomic energy (which we hope will be abundantly available). Cultures of hydrogenomonads seem to lend themselves admirably to industrial conversion of inorganic to organic matter.

Bioexchanger (that is, alga) programs started during and after World War II, when the need for a greater food supply was felt acutely, even in the industrialized nations. A few years later, however, these nations were fighting a food surplus and it became clear that pills and plows might serve hungry nations better than sophiticated food plants. For a while, bioexchangers were seriously considered for submarines, but more ready solutions have been used instead. Space exploration presented new possibilities. Dr. Jenkins' impressive projections concerning size and duration of not-too-distant missions may well indicate that the bioexchanger is the only reasonable solution.

The outlook is improved, although we must not forget earlier frustrations due to haphazardly planned experiments, diversions from key issues, and lack of integration between scientist and engineer.

Are bioexchangers feasible? I believe they are; but am not yet ready to prove this to the engineer or (which is more important), to the astronaut. As far as I know, cultures of algae or hydrogenomonads have never been maintained in a realistic configuration at zero gravity. Long-term closed-cycle operation and nutritional aspects must be clarified further before the bioexchanger can be presented with full confidence. This cannot be long delayed, because the subsequent development of a full-fledge spaceborne exchanger will be long and complicated.

Beyond a certain intensity, photosynthesis does not accelerate with increasing light, since temperature-dependent, enzymic processing steps can only keep pace with a certain limited flux of quanta into the photochemical conversion centers. Intensities beyond this are actually dangerous and seem to require protective devices in the photosynthetic apparatus (refs. 1–3). These effects underly the relatively low efficiency of algal cultures that use bright illuminators or natural sunlight (refs. 4, 5). This brings up one aspect of photosynthesis in bright light: the locus of the rate-limiting dark step in the electron transport sequence.

The stage was set for the search for the limiting step in photosynthesis by the experiments of Emerson and Arnold in 1932 (ref. 6); they gave series of brief, bright flashes to a suspension of algae, and measured the oxygen evolved per flash as a function of the time between the flashes. The result is well known: in a single flash—no matter how bright—no more light can be converted than corresponds to one oxygen per $\simeq$2500 chlorophyllis; i.e., only one quantum can be "caught" per some 250 chlorophylls (assuming that 10 quanta are needed to generate one oxygen). About half this maximum amount of oxygen was evolved in case the flashes were spaced $\sim$10 msec apart. Thus, if we assume the limiting step to be a first-order reaction in which an enzyme E transfers an electron, the maximum rate is $k_d \times [E_{tot}]$, k_d being the reciprocal of 10 msec and the total concentration of the limiting enzyme E being $\sim$1/250 chlorophyll. These experiments led to the concept of

the photosynthetic unit: groups of ~250 chlorophylls acting like one giant pigment molecule which drains its absorbed energy into a single trapping and conversion center. At present we know that there are two photoacts—each having its own pigment complex and trapping center. More refined experiments with repetitive flashes, exemplified in figure 1, showed that the assumption of a single first-order limiting step might be too simple, and fitted better with the assumption of two steps which, in conjunction, limit the rate. Apparently the two slowest reactions in the chain (which could be at entirely different loci), have nearly equal transfer rates. One of the two reactions took less time (1 to 5 msec) and the other more time (20 to 50 msec) than the above mentioned 10-msec halftime. The kinetic data discussed so msec. halftime. The kinetic data discussed so far (obtained a decade or more ago with whole cells) do not indicate which of the numerous steps in photosynthesis are responsible. Recent work with isolated chloroplasts and more sophisticated techniques may yield this information.

Figure 2 illustrates the electron transport chain in photosynthesis, as conceived at present. Water is split into oxygen and reducing power by means of two series-connected photoreactions. Each photoact causes the transfer of an electron from a substance with a more oxidizing potential to one with a more reducing potential, so that chemical work is done by the light. One photosystem (system II) produces a strong oxidant that evolves oxygen, and a weak reductant (Q). The other, photosystem I, produces a weak oxidant (P) that oxidizes the weak reductant of system II, and at the same time a very strong reductant capable of reducing ferredoxin and, in the whole cell, carbon dioxide. The thin arrows in figure 1 symbolize three sets of dark reactions: k_1 is the dark process in which the oxidizing power, generated in photoact II, liberates oxygen, k_2 symbolizes the reaction chain which interconnects the two photoacts. k_3 symbolizes the dark reaction which the strong reductant of system I undergoes. Whereas, in whole cells, reaction k_3 is an extremely complex process (the Calvin-Benson cycle—ref. 8), in isolated chloroplasts many artificial electron acceptors can react directly with the primary photoreductant of system I (X in fig. 2). Only the reduction of NADP requires the mediation of two chloroplasts enzymes, the non heme protein ferredoxin and the flavoprotein NADP reductase (ref. 9). Dyes which undergo a color change upon oxidation or reduction have been

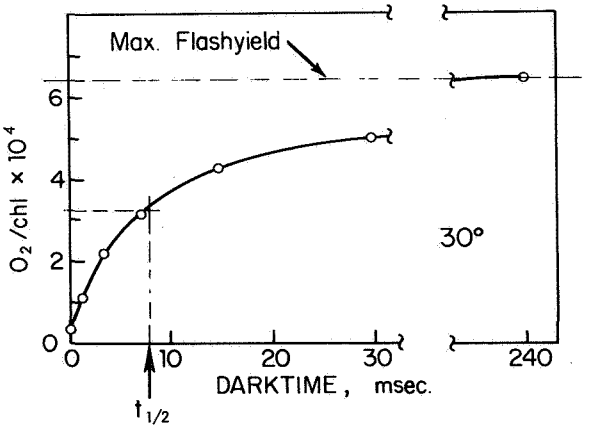

FIGURE 1.—Yield of O_2 per flash as a function of dark time between flashes observed with whole Scenedesmus cells at 30°C (ref. 7).

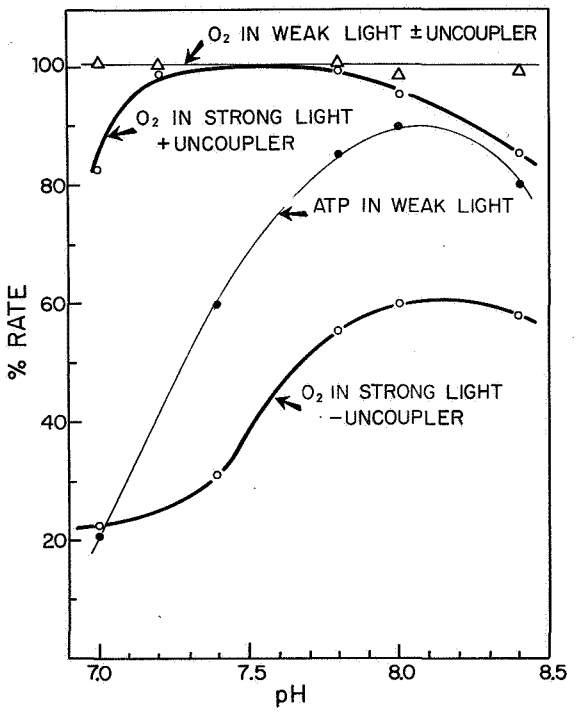

FIGURE 2.—Top: schematic representation of photosynthetic electron transport, driven by two series-connected photoacts. Bottom: simplified reaction chain between the two photoacts.

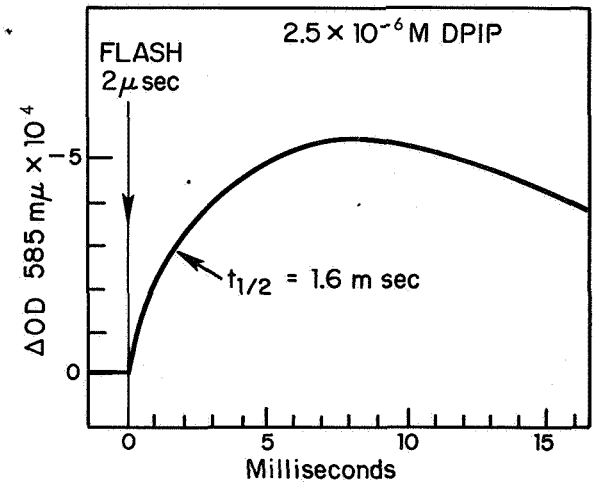

FIGURE 3.—Spectroscopic observation of the flash-induced reduction by isolated chloroplasts of DCPIP, present in a concentration of 2.5×10^{-6}M. Reduction halftime decreases proportionally with dye concentration (ref. 11).

used recently in our laboratory by Forbush to measure the rates of their photoreduction by chloroplasts in step k_3. Using sensitive spectroscopy he found that such dyes, used in optimal concentrations, were reduced with extreme rapidity after he gave brief flashes of strong light. Halftimes of 0.1 to 1 msec were observed (fig. 3). However, when these dyes are used as electron acceptors in isolated chloroplasts, the rate of oxygen evolution is not higher than the rates observed with slower acting oxidants or, in fact, with whole algae using CO_2 as the ultimate oxidant (~1000 eq/Chl hour). It is obvious therefore, that the rate-limiting dark step in photosynthesis should not be sought in reaction k_3 or the carbon-dioxide reduction path. On the other hand, "air breathing" land plants must cope with the very low CO_2 concentration in the atmosphere so that the rate of CO_2 supply is the limiting step if all other supplies are abundant (ref. 10). In algal cultures, plenty of CO_2 or bicarbonate can be provided so that this aspect is not important.

Our search for the internal rate-limiting step is thus facilitated, because we can study isolated chloroplasts, which are more amenable to biochemical analysis than the whole cell.

Of the two remaining dark processes shown in figure 1, recent work by Joliot (ref. 12) yielded direct information concerning step k_1: the evolution of oxygen. A polarograph was used with a bare platinum electrode upon which a thin layer of algae or chloroplasts was deposited. With an appropriate polarization voltage between the platinum and a reference electrode, the current flowing between the two electrodes is proportional to the O_2 tension. By using an intermittent light beam to excite photosynthesis and a tuned ac amplifier to measure the current, one can view exclusively the pulsating evolution of oxygen induced by the pulsating light. By varying the modulation frequency and analyzing the phase delay between the light pulses and the oxygen pulses, it proved possible to measure the time constant for the O_2 evolution reaction. Joliot observed a delay between light absorption and appearance of O_2 of 0.9 msec (at 27°C). This measurement was made in weak light and cannot be extrapolated to conditions of light saturation. It is still possible that one of the rate-limiting steps discussed above is in reaction k_1, the formation of oxygen. If so, the slower limiting step should be sought in the connective link between the photoacts (k_2 in fig. 2). If the O_2-evolving reaction itself is never limiting, all rate limitation must be sought in this chain.

As recent reviews of photosynthesis reveal, we generally assume that reaction k_2 is very complex. It involves several intermediates and might be coupled with the generation of ATP from ADP. Hill and coworkers (ref. 13) who discovered the chloroplast cytochromes *f* and *b*, hypothesized that photosystem I oxidized cytochrome *f* and photosystem II reduced cytochrome *b*, while the subsequent oxidation of ferrocytochrome *b* by ferricytochrome *f* yielded ATP. Whether this specific hypothesis is correct is immaterial for the present discussion, in which we assume that only a single phosphorylation site occurs.

As was shown by Arnon et al. (ref. 14), freshly isolated chloroplasts show a rather tight "coupling" between electron transport and generation of ATP (fig. 4). That is, in *strong* light the rate of electron transport is depressed if phosphorylation cannot occur, either by lack of substrate (ADP, etc.) or, for example, by an

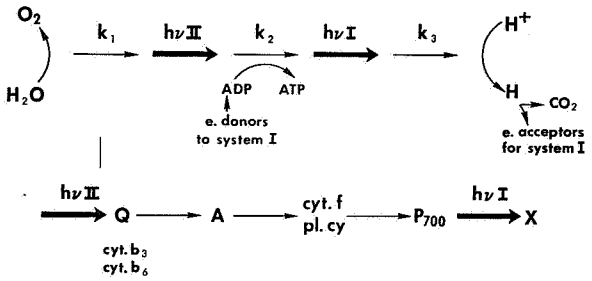

FIGURE 4.—Dependence of the rate of O_2 evolution and concomitant ATP formation in weak light (thin curves) and of the rate of O_2 evolution in strong light, in the presence or absence of 10^{-3}M ammonium ion, which uncouples the phosphorylation step. Spinach chloroplasts (ref. 15).

unfavorable pH, as shown in figure 4. At the correct pH, the addition of ADP enhances the maximum rate in strong light. One can also add an uncoupling agent, such as ammonium ion which prevents ATP formation and at the same time releases the braking effect of the frustrated phosphorylation step.

It has been asked whether the so-called "basal" electron transport, which is unaffected by the phosphorylating step, occurs via a "bypass," parallel to the coupling step, or via the coupling step itself but at a slower pace. The fact that over a considerable range of weak intensities the rate (qauntum yield) of electron transport is independent of phosphorylation suggests the latter interpretation. The fact that under some conditions the phosphorylating site can severely limit the rate does not necessarily imply that under uncoupled or phosphorylating conditions the same site sets the pace. Probably another step is now the bottleneck.

Avron (ref. 16) found that under appropriate conditions light-driven ATP formation can occur at very high rates, much higher than what correspond to the maximum rate of O_2 evolution observed in choroplasts (>2000 eq. ATP/Chl hour compared with <1000 eq. O_2/chl. hour). Thus the phosphorylation site itself can operate very fast.

These high rates of photophosphorylation can be seen when only photosystem I seems to be operative. Similarly high rates of photosystem I can be observed in terms of electron transport. In these cases artificial electron donors instead of H_2O are used to feed electrons to the photooxidant of system I; system II being inhibited by aging, detergent or an inhibitor like DCMU (ref. 17) (see fig. 2). Izawa et al. (ref. 18) recently observed, in fresh DCMU-poisoned chloroplasts, a rate of electron transport of as high as ~6000 eq/Chl hour, simultaneous with a phosphorylation rate of ~900 moles ATP/Chl hour. Obviously we must seek the bottleneck in photosystem II or a closely associated step. In figure 2, Q indicates the as yet unidentified primary photoreductant of system II (a quantum causes the transfer of an electron to Q). Symbol Q was chosen by Duysens (ref. 19) because, in its active, oxidized state, it quenches the fluorescence of the pigment that sensitizes system II. Q cannot receive another quantum unless restored (oxidized) in reaction k_2 so that, with Q in the reduced form, incoming quanta have nowhere to go and will probably escape from the sensitizing pigment as fluorescence. Thus, the fluorescence yield of system II pigment can serve as an indicator of the redox state of Q.

In figure 2 the symbol P is used for the photooxidant of system I. This special long-wave-absorbing chlorophyll complex (P700) occurs in about the same low concentration as Q: ~1/500 chlorophylls. In photoact I, an incoming quantum travels to P700, which absorbs strongly at 700 mμ as long as it is in its reduced state. Upon excitation the pigment loses an electron (to X in fig. 2) and is bleached. With sensitive spectroscopic equipment one can measure the degree of bleaching.

Using these two methods to observe the redox states of P and Q, one observes that in bright, saturating light all Q becomes reduced and all P oxidized. The electron transfer from Q to P (k_2 in fig. 2, top) apparently is to slow to keep pace with the light flux and the other faster, dark reactions, and thus is rate-limiting. Actually, P and Q do not react directly with each other; two or three other intermediates are involved. One of these, denoted in scheme 2 as A (plastoquinone?) occurs in much larger (~10 X) concentration than P and Q; it acts like a buffer pool between the two photoacts. It could be shown (refs. 20 and 21) that in strong light A, like E, becomes fully reduced. This proved that the electron transfer from E to A

is faster than that from A to P. Recent experiments (ref. 11) indeed revealed that the transfer E to A was quite fast (<1 msec with all A in the oxidized form). This would leave the transfer from A to P as the limiting reaction. In a not fully clarified fashion two enzymes, cytochrome f and plastocyanin, operate between these two compounds. Electron transfer from cytochrome f to P appears extremely rapid (<1 msec) (ref. 22) and, although no direct observations are available, the same might well be true for the reduction of P^+ by plastocyanin (ref. 23). Thus we can confine the limiting step to the transfer from pool A to cytochrome f and/or plastocyanin. Unfortunately, this conclusion leads to more problems and inconsistencies. The reactions "between" the photoacts—i.e. the events occurring in the medium potential region—must be quite complex; besides the discussed intermediates, two b cytochromes and possibly other electron carriers are involved. Interaction with system II may be more intimate than was supposed, and our linear scheme may be wrong. Much of our present work is concentrated on this important reaction sequence; it will take time to understand it.

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COMMENTS

Dr. KRAUSS. Have you looked at the differences in the kinetics as your Chlorophyll A–B ratios shift? Are they fairly constant?

Dr. KOK. No. We have seen chains of the A and B ratio but have never made systematic observations. Other people have been doing this in different ways by separation. Nowadays, the chlorophyll can be separated in test tubes, more or less. After you treat it with a little detergent, you can spin and get original system I and II; ratios up to 15 have been obtained in this fashion. Your procedure may well be preferable.

Dr. KRAUSS. I do not know that it is preferable, but it might be useful to compare the results.

Ecologic Relationships Between Bacteria and Algae in Mass Culture

C. H. WARD
Rice University
Houston, Texas, and
J. E. MOYER
Robert S. Kerr Water Research Center
Ada, Oklahoma

Interactions between bacteria and algae undoubtedly affected the physiology and productivity of aquatic communities (refs. 1 and 2). Bacteria have been reported to enhance algal growth (refs. 3 and 4) but are generally believed to have little or no effect on algal productivity under conditions otherwise optimum for growth (refs. 5–7). In contrast, algae have been shown to both stimulate (ref. 2) and inhibit (ref. 8) growth of associated bacteria. These diverse relationships appear to be species contingent and influenced to a large degree by the chemical and physical environment. Soluble organic compounds, as found in lakes and streams, support the growth of heterotropic bacteria and serve to complicate the ecological and biochemical relationships involved. Seasonal fluctuations in temperature and rainfall are probably modifying parameters.

Our interest in algal-bacterial interactions was prompted by the knowledge that laboratory cultures of unicellular green algae, grown autotrophically in liquid inorganic media, frequently become heavily contaminated with bacteria. Contaminant populations often reach levels of 10^6 to 10^9 viable bacteria per ml of culture, yet little significance has been attributed to contamination and it is frequently ignored. Krauss and Thomas (ref. 9) have suggested that contaminant bacteria grow on algal cell wall debris and possibly algal excretory products.

Our studies were initiated to elucidate the role of contaminant bacteria in mass cultures of algae used for photosynthetic gas exchange. Although quantitative data are lacking, it is believed by some investigators that bacterial contaminants are responsible for, or are associated with, a variety of algal mass-culture maladies variously described as foaming, fouling, and sticking.

This paper presents results of NASA-supported research (NASA-Defense PR No. R–99) on algal-bacterial relationships conducted at the USAF School of Aerospace Medicine, Brooks Air Force Base, Texas. The early phases of this investigation have been reported (refs. 10–12); a final report and manuscripts dealing with identification and bacterial utilization of algal excretory products are in preparation (refs. 13–15). Hence, detailed experimental procedures will not be given in this paper.

All experimentation was done using the thermotolerant (39°C) alga *chlorella pyrenoidosa* TX71105 (ref. 16), which recently has been renamed *Chlorella sorokiniana* (ref. 17). Algal cultures were grown on inorganic media (Knop's) in illuminated water baths, annular chambers, thin-panel mass-culture devices, and constant temperature incubator-shakers. Cultures were illuminated with flourescent lamps and aerated with carbon-dioxide enriched air (1 to 5 percent). All experiments were performed with axenic algal cultures unless deliberately contaminated with selected bacteria. Bacteria were isolated and enumerated using standard bacteriological procedures.

BACTERIAL CONTAMINANTS OF ALGAL CULTURES

Krauss and Thomas (ref. 9) found that a bacterium probably belonging to the genus *Flavobacterium* was most common in cultures of *Scenedesmus obliquus*. A *Flavobacterium* species was also found to be prevalent in cultures of *Chlorella vulgaris* (ref. 18).

Isolations from our algal mass cultures re-

vealed a variety of bacterial forms, with several occurring in large numbers. In an effort to obtain a better representative sampling of the types of bacteria that inhabit algal cultures, samples of TX71105 cultures were obtained from five other laboratories. Results of our isolations are presented in table I. Only bacteria occurring in large numbers were identified. All species isolated were heterotrophic. It is evident that only a limited number of bacteria reach large populations in algal cultures. It is also significant that *Pseudomonas aeruginosa* and *Mima polymorpha* were isolated from all cultures examined.

TABLE I.—*Bacteria Isolated from Mass Cultures of* Chlorella Pyrenoidosa *TX71105 (ref. 10)*

Source	Organisms isolated
USAF School of Aerospace Medicine, Brooks AFB, Tex.	*Ps. aeruginosa.* *Mima polymorpha.* Gram-negative bacillus (yellow pigment). *Bacillus* sp.
Martin Company, Denver, Colo.	*Ps. aeruginosa.* *Mima polymorpha.* *Bacterium anitratum* (typical strain). *Staph. epidermidis.* *Serratia marcescens.*
University of Maryland, College Park, Md.	*Ps. aeruginosa.* *Mima polymorpha.* *Bacterium anitratum* (atypical strain). *Aerobacter cloacae.*
Armed Forces Food & Container Institute, Chicago, Ill.	*Ps. aeruginosa.* *Bacterium anitratum* (typical strain). *Bacterium anitratum* (atypical strain). Gram-negative bacillus (yellow pigment).
General Dynamics/Electric Boat, Groton, Conn.	*Ps. aeruginosa.* *Mima polymorpha.* *Bacterium anitratum* (typical strain). *Bacillus* sp. *Aerobater cloacea.* *Aerobacter aerogenes.*
U.S. Naval Research Lab., Washington, D.C.	*Ps. aeruginosa.* *Mima polymorpha.* Gram-negative bacillus (yellow pigment). *Aerobacter cloacae.* *Aerobacter aerogenes.*

In other experiments in this series (ref. 11), it was shown that most soil and air bacteria do not survive when inoculated into axenic algal cultures. However, two enteric pathogens, *Salmonella typhi* and *S. paratyphi*, grew well in algal cultures for prolonged periods. Thus, a highly selective mechanism appears to be operative, and the unexpected growth of human pathogens suggests that it may be necessary to separate the biological components of regenerative life-support systems.

GROWTH OF BACTERIA IN ALGAL CULTURES

Earlier work with large (8-liter) batch algal cultures by Ward et al. (ref. 19) showed what

appeared to be a direct relationship between growth of algae and contaminant bacteria. Experiments performed with smaller, more manageable cultures, deliberately contaminated with selected bacteria, have served to clarify this relationship.

When dilute algal cultures are exposed to light of saturating intensity (about 2000 ft-c), growth is exponential. Figure 1 shows that contaminants in light-saturated cultures also grow exponentially. Algal cultures grown under light-limited conditions demonstrate linear growth where the increment of cell increase is constant with time. Contaminants in light-limited algal cultures also increase at a linear rate (fig. 2). These data confirm and extend earlier work (ref. 19) and clearly demonstrate the dependence of bacterial growth on algal growth. These data also suggest, but do not demonstrate, a nutritional relationship between bacteria and algae in mixed culture as proposed by Krauss and Thomas (ref. 9). A nutritional or pathogenic relationship would appear necessary because, with the exception of EDTA (ethylenediamine tetraacetic acid) used for chelation of trace elements, the algal medium contained only inorganic salts and distilled-deionized water. Only heterotrophic bacteria were present in the algal cultures and, hence, required a source of fixed carbon for growth. Blasco (ref. 20) has proposed a pathogenic relationship to explain growth of bacteria in algal cultures.

Figure 3 illustrates the different patterns of bacterial growth when present as single contaminants in algal batch cultures. Bacterial growth patterns undoubtedly reflect those of algal growth to some extent since the experiments were done on different days. However, total algal growth for all control experiments was about 3.5 mg/ml, indicating that bacterial growth is dependent on both the species of bacteria involved and the amount and habit of algal growth. Concurrent growth of the five bacteria in a batch culture of TX71105 clearly indicates dominance of some bacterial species (fig. 4).

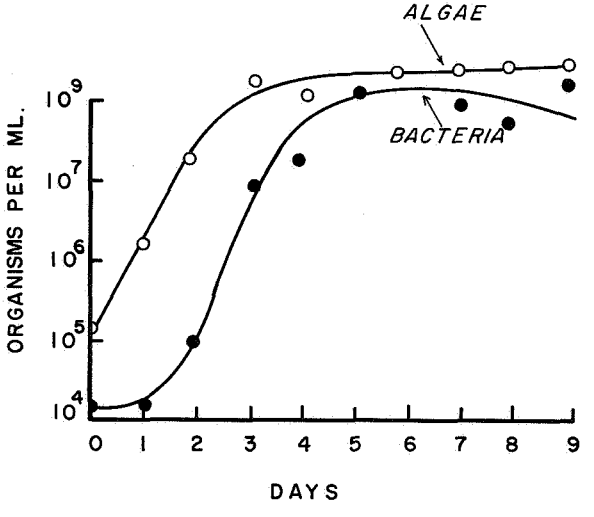

FIGURE 1.—Growth curves of Chlorella pyrenoidosa TX71105 and a gram-negative bacillus added to the culture as a bacterial concomitant (ref. 11).

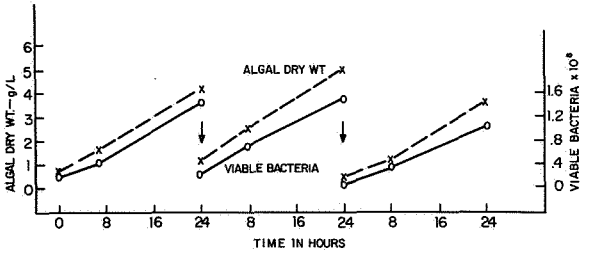

FIGURE 2.—Growth of Chlorella pyrenoidosa TX71105 and selected bacteria in mass culture (ref. 10).

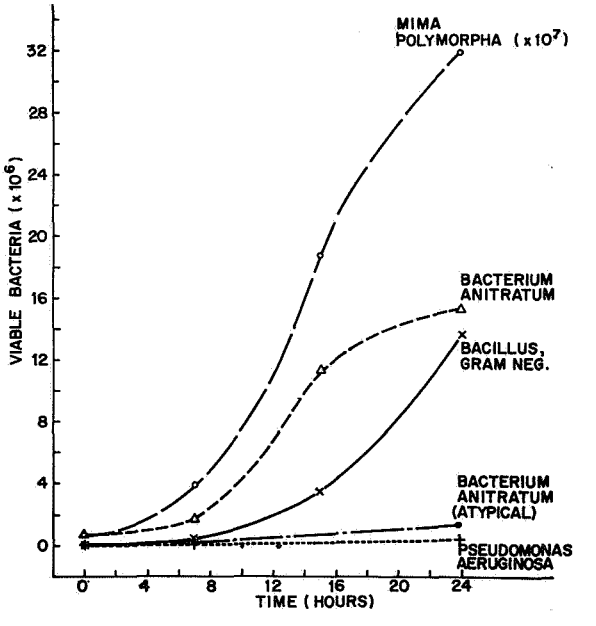

FIGURE 3.—Growth of bacteria in algal cultures (ref. 10).

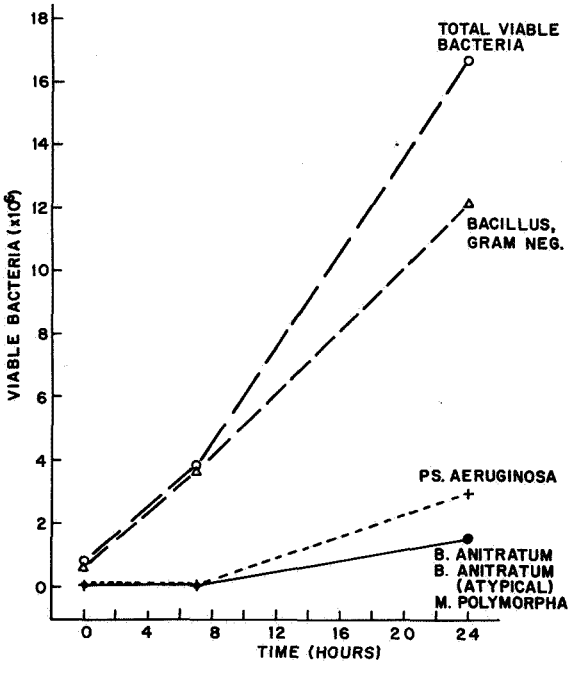

FIGURE 4.—Concurrent growth of five bacteria in a culture of Chlorella pyrenoidosa TX71105 (ref. 10).

B. anitratum, *B. anitratum* (atypical), and *M. polymorpha* cannot be readily distinguished on the basis of colonial form; hence, the total count for the three is given. The gram-negative bacillus appears to have the greatest competitive advantage. Of special interest is that *M. polymorpha* grew poorly in association with other bacteria but reproduced profusely when present as a single contaminant. These experiments suggest that interspecies antagonism may also be involved in the growth dynamics of bacteria in algal cultures.

To determine if contaminant bacteria influence the growth of algae, growth of contaminated batch cultures was compared to that of uncontaminated controls (table II). Data for algal growth are expressed as percent of controls. Bacteria had a minor effect on the optical density of algal cultures. However, four of the six bacteria reduced algal cell number by about 20 percent and caused a 5- to 13-percent decrease in culture dry weight. The bacterial contribution to culture mass averaged 1 percent or less. *Ps. aeruginosa* reduced algal growth even at low concentrations; however, the combined effects of five bacteria growing in the same culture did not exceed that of any detrimental bacterium acting independently. These data quantitatively show the adverse effects of certain bacteria on algal growth. One can only speculate about the mechanism involved; however, it is significant that repeated microscopic examinations revealed no evidence of an infectious pathogenic relationship.

Growth of Bacteria in Algal Culture Filtrates

Because no evidence of parasitism or infectious disease was observed, experiments were performed to determine if algal cell walls released during cell division or products excreted during growth serve as nutrient for growth of contaminant bacteria. In addition, the possibility exists that algae excrete bacterial metabolites in response to the presence of bacteria. If

TABLE II.—*Effects of Bacteria on the Growth of* Chlorella Pyrenoidosa *TX71105, Bacterial Growth in Algal Cultures, and Bacterial Contribution to Culture Mass (ref. 10)*

Bacterium	Algal growth with bacteria[a]			Viable bacteria	
	O.D.	Cell no.	Dry wt.[b]	No. × 10^6/ml	% Dry wt.
Mima polymorpha	104	80	95	311. 3	1. 03
Bacillus, gram neg	—	80	91	13. 6	0. 71
Bacterium anitratum	89	77	87	14. 5	0. 98
Bacterium anitratum (atypical strain)	98	100	102	1. 2	0. 56
Pseudomonas aeruginosa	101	80	89	0. 4	0. 01
Aerobacter cloacae	103	101	103	22. 2	0. 25
Combined bacteria[c]	91	85	88	16. 1	0. 93

Data are means of six or more replicates corrected for initials and—
[a] expressed as percent of bacteria-free controls;
[b] corrected for contribution of bacteria;
[c] first five bacteria listed.

algae normally excrete soluble organic materials, the effluent from bacteria-free algal cultures should contain substances oxidizable by heterotrophic bacteria.

Sterile, cell-wall-free, algal effluent was prepared from 24-hour axenic cultures by centrifugation and passage through 0.45-μ membrane filters. Test bacteria were grown on trypticase soy broth, centrifuged, washed twice with saline, and starved for varying periods of time depending on the experiment.

Figure 5 shows typical results obtained by Warburg respirometry. Similar results were obtained with *M. polymorpha*, *B. anitratum*, and

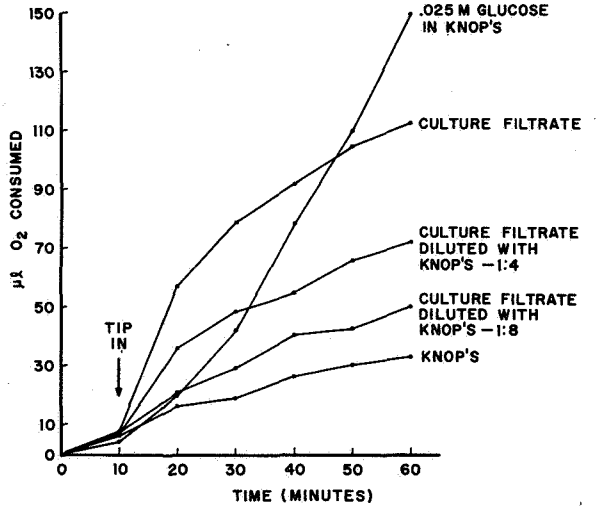

FIGURE 5.—Oxidation of culture filtrate from axenic Chlorella pyrenoidosa TX71105 cultures by Pseudomonas aeruginosa (ref. 10).

B. anitratum (atypical). The presence of substrates in algal effluent oxidizable by the test bacteria is clearly evident. A typical growth curve is shown in figure 6. All six of the bacteria tested (see table II) increased by at least two logs in 8 hours in 20-ml portions of the algal effluent. The small increase observed in Knop's (control) proved to be caused by stored food reserves. Experiments to determine if EDTA could serve as a carbon source for the test bacteria were negative.

Another experiment in this series (ref. 11) was designed to determine if bacteria selectively remove certain compounds when growing in algal cultures, while leaving others subject to oxidation by different bacteria. Algal cultures

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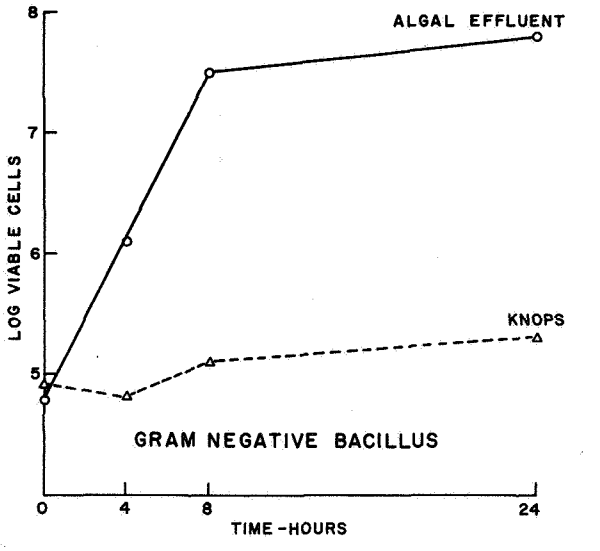

FIGURE 6.—Growth of gram-negative bacillus in effluent Knop's solution from 24-hour axenic Chlorella pyrenoidosa TX71105 cultures.

were grown in combination with the test bacteria and the presence of oxidizable materials in culture filtrates determined by Warburg respirometry (table III). It is evident that at least two or more substances are excreted by TX71105 since the gram-negative bacillus oxidized substances not subject to breakdown by the other bacteria tested. The presence of several types of excretory products could explain differences in competitive advantage observed in the growth experiments.

Identification of Algal Excretory Products

Because previous experiments showed that soluble substances of algal origin serve as nutrient for growth of contaminant bacteria, it appeared important to identify these substances as a prelude to evaluating their utilization by bacteria.

Fogg (refs. 1 and 21) has reviewed available information on the extra cellular products of algae. Marine phytoplankton have recently been shown to excrete up to 25 percent of their photoassimilated carbon during logarithmic growth (ref. 22). The excretion of glycolic acid by *C. pyrenoidosa* during photosynthesis has been extensively studies by Tolbert and Zill (ref. 23). Several organic acids have been reported as algal extracellular products. Goryunova (see

TABLE III.—*Selective Utilization of Oxidizable Substances by Bacteria Growing in Algal Cultures (ref. 11)*

Filtrates from 4-day cultures of the following:	Ratio of oxygen consumed by these test bacteria [a]			
	BAT [b]	MP [b]	BAA [b]	GNB [b]
Axenic Chlorella TX71105	0.60	0.47	0.58	0.68
TX71105+BAT	0	0	0	0.16
TX71105+MP	0	0.03	0.03	0.16
TX71105+BAA	0	0.02	0	0.37
TX71105+GNB	0.12	0.07	0.15	0
TX71105+all 4	0	0	0.01	0

[a] Ratio of μl O_2 consumed by 10^9 starved bacteria in 20 min. Ratio represents the amount of oxygen utilized by test bacteria using culture filtrate as substrate and 0.025 *M*-glucose as substrate. The results were corrected for endogenous respiration as measured by using Knop's medium:

$$\frac{\text{Culture filtrate-Knop's medium}}{\text{0.025 } M\text{-glucose-Knop's medium}}$$

[b] BAT, *Bacterium anitratum* (typical); MP, *Mima polymorpha;* BAA, *Bacterium anitratum* (atypical); GNB, gram-negative bacillus.

ref. 1) found oxalic, tartaric, succinic, and other organic acids in filtrates from cultures of *Oscillatoria splendida.* Appreciable quantities of glycolic, oxalic, and probably pyruvic acids were reported by Allen (ref. 24) to be excreted by various species of *Chlamydomonas.* Other algal excretory products, such as amino acids and peptides, carbohydrates, vitamins and growth substances, inhibitors and antibiotics, toxins, and enzymes have been reported (ref. 1). The literature concerning algal excretions is inconsistent; yet there appear to be certain species specificities with respect to the substances excreted as extracellular products.

Axenic algal cultures were grown in 400 ml of sterile Knop's medium in 1-liter Erlenmeyer flasks. Flasks were held at 37° in a Model B–27 New Brunswick incubator-shaker and illuminated from beneath at about 2000 ft-c with fluorescent lights. A gaseous atmosphere containing 4-percent carbon dioxide was maintained. Dense cultures were transferred to sterile 200-ml centrifuge tubes and centrifuged for 40 minutes at 7000 rpm while being maintained at 2°C. The supernatant fluid was then passed through 0.45-μ membrane filters. For 36 hours, 100-ml samples of culture filtrate were dialyzed at 5°C against five changes (350 ml each) of distilled, deionized water. The dialysate was collected and stored at 5°C and subsequently concentrated in a rotary evaporator to less than 100 ml. Fractionation schemes for the concentrated dialysates are shown in figures 7 and 8.) After dialysates were separated into various components, the fractions were evaporated to 2 ml in a rotary evaporatory. Standard paper chromatographic procedures were used for qualitative identification of substances present in the various fractions (refs. 14 and 15). Some amino-acid analyses were performed using the Technicon Amino-Acid Analyzer. Other standard analytical methods were used as needed. Table IV lists the major constituents identified in axenic culture filtrates of *Chlorella* TX71105. Several of the

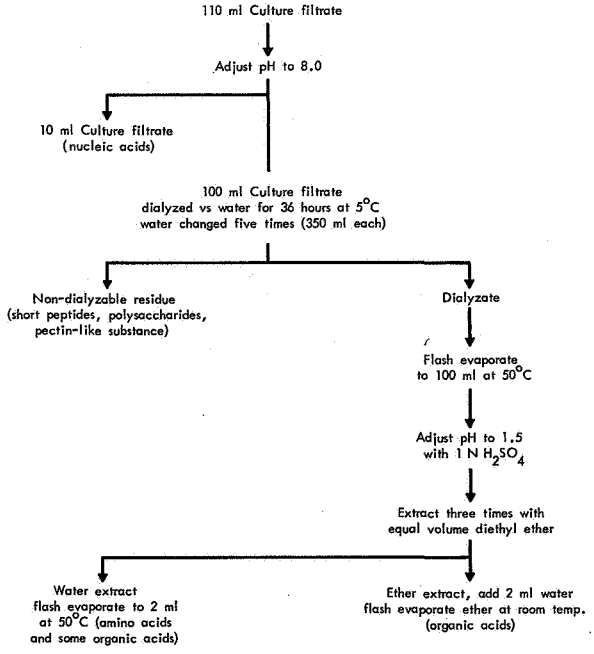

FIGURE 7.—Fractionation scheme I.

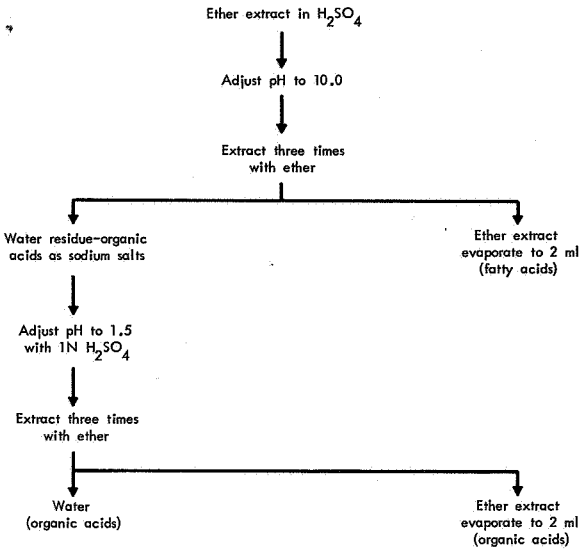

FIGURE 8.—Fractionation scheme II.

compounds detected were not identified. Quantitation of organic acids was accomplished with great difficulty and with less precision than desired. The high molecular-weight groups of compounds found in the nondialyzable fraction were not further identified. Many of the substances identified are known as sources of carbon and energy for the growth of heterotrophic bacteria.

Utilization of Algal Excretory Products by Selected Bacteria

Utilization of algal excretory products was studied using four bacteria growing singly and in combination in axenic culture filtrates. Culture filtrates were prepared as prevously described from algal cultures containing approximately 10^8 cells/ml. Test bacteria were grown on trypticase soy broth as previously described and starved for 2 hours in 0.9-percent saline solution. Inocula of 10^3 viable cells of each bacterium were added singly and in combination to separate 200-ml portions of axenic culture filtrate and incubated with agitation for 24 hours at 37°C. Bacteria were separated by centrifugation and filtration. Metabolized filtrates were fractionated for analyses using procedures shown in figures 7 and 8.

Organic acids were assayed spectrophotometrically. Glycolic acid was measured using 2, 7-naphthalenediol for color development. Precision of the technique was severely limited by interferences caused by the presence of other organic acids. The standard addition technique was only partially effective, depending on the types of organic acids present. As a consequence, only data for lactic acid and glycolic acid will be presented.

TABLE IV.—*Extracellular Products of* Chlorella Pyrenoidosa *TX71105 Present in Axenic Culture Filtrates*

Organic acids	Amino acids	Other
Fumaric.	Aspartic.	Polysaccharides.
Lactic.	Glutamic.	Short peptides.
Glycolic.	Serine.	Nucleic acids.
Oxalic.	Threonine.	Ammonia.
Pyruvic.	Isoleucine.	
α-ketoglutaric.	Leucine.	
Oxalacetic.	Tyrosine.	
Ascorbic.	Phenylalanine.	
Gluconic.	Lysine.	
Galacturonic.	Proline.	
	Alanine.	
	Glycine.	
	Cystine.	
	Valine.	
	Histidine.	
	Ornithine.	

Ninhydrin-positive substances were assayed using the Technicon Amino-Acid Analyzer. Several substances that appeared to be peptides could not be definitely identified. One peptide-like substance occurred in large quantities and was only slightly metabolized by the test bacteria. Other unknowns occurred in trace amounts only. Quantitation of the amino acids was relatively good. Summary data on 11 amino acids and two organic acids are presented (table V).

With the exception of the organic acids, *B. anitratum* (typical) and *B. anitratum* (atypical) utilized algal excretory products to about the same extent. The atypical strain utilized both lactic acid and glycolic acids; however, these acids were not metabolized by the closely related typical strain. *M. polymorpha* failed to use either organic acid, but the gram-negative bacillus utilized all of the lactic acid and apparently excreted glycolic acid. The gram-negative bacillus also failed to utilize leucine and isoleucine but was the only bacterium that removed the trace of lysine present in the culture filtrates. Although data are incomplete, assays for total organic acids indicated that utilization of amino acids by the test bacteria was remarkably greater than utilization of organic acids. The ecological significance of this finding is not readily apparent. However, it should be noted that the combined activities of the four bacteria served essentially to eliminate the products excreted during algal growth. This finding was previously demonstrated in the manometry studies (table III) and may explain the tendency of algal cultures to support only a few types of bacteria when unprotected from outside contaminants. If the contaminant flora removes excretory products as rapidly as they are released by growing algae, contamination by other heterotropic bacteria would seem unlikely. Thus it may be possible to establish a known bacteria flora in algal cultures that would effectively prevent invasion by less desirable (more harmful) forms and hence improve stability. It now appears that the observed detrimental effects of bacteria on algal growth may be caused only by their utilization of the products excreted. This would represent loss of carbon and efficiency if algae normally reabsorb extracellular materials. Bacterial products may also be toxic to algae. However, we found urea, a known nitrogen source for *Chlorella* TX71105, to be a major excretory product of three of the four test bacteria. Other bacterial products identified were methionine, tryptophan, and glucosamine.

Contaminated algal mass cultures represent complex ecologies that can be subjected to

TABLE V.—*Utilization of Excretory Products of* Chlorella Pyrenoidosa *TX71105 by Selected Bacteria*

Compound	Conc. in algal filtrate μg/ml	Relative utilization [a]				
		BAT	BAA	MP	GNB	Combined
Aspartic acid	13.3	+++	+++	+	+++	+++
Threonine	4.8	+++	+++	++	+++	++
Serine	5.3	+++	+++	++	+++	+++
Glutmaic acid	11.8	+	+	++	−[b]	+++
Proline	3.7	+++	+++	+++	++	+++
Alanine	5.3	+++	+++	++	+++	+++
Isoleucine	2.1	+++	+++	++	−	+++
Leucine	1.9	+++	+++	+++	−	+++
Tyrosine	trace	+++	+++	−	−	+++
Phenylalanine	1.2	+++	+++	+++	++	++
Lysine	trace	−	−	−	+++	+++
Lactic acid	1.1	−	+++	−	+++	+++
Glycolic acid	1.3	−	+	−	−[b]	+

[a] Data are relative: +++ indicates complete removal; ++, trace remaining; +, one half or more remaining; −, no utilization.
[b] GNB apparently excreted small quantities of these compounds.

quantitative investigation. Investigations of the type reported should be extended to the study of natural systems and to the complex interactions that undoubtedly occur in mass cultures of other organisms being considered for bioregeneration, e.g., *Hydrogenomonas* spp.

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COMMENTS

Mr. BEEM. Did you make any observations of the foaming characteristics?

Dr. WARD. No. I think there is qualitative evidence to support the fact that bacteria are associated with foaming. Dr. Krauss does not have problems with foaming, and he runs clean systems. I think there is ample evidence. No one really quantitated it to that extent to show this is definitely the case, but we have pretty well gone on the assumption.

Dr. MILLER. In the continuous culture systems that we have, which are run uncleaned, they are contaminated. In some cases they are fairly heavily contaminated, but we find foaming only during the initial portion of the growth curve where you are growing up to the population density which you are going to control. After you have reached the point in the cultures in that state, foaming does not appear to us to be as much of a problem. We do not get foaming in an actively growing-state culture.

Dr. COOKE. Do you ever find enhancement of algal growth due to bacterial contamination?

Dr. WARD. No, but it has been reported. The Russian workers reported it several times. They get beautiful enhancement. We have seen not one indication of any type of enhancement.

Dr. GOLDNER. Did you note whether the bacterial contamination produced any changes in your algal cultures?

Dr. WARD. We routinely monitored pH. I do not believe that there was a real effect on the pH. You probably could measure one, but it is not a real significant effect.

Dr. GOLDNER. Did you note any morphologic relationship between the bacterial contaminants and the algal cells? For example, did you observe embedment of the bacterial cells on the cell wall of the algae cells?

Dr. WARD. No. This is what we did not see, and this is why I indicated that some peope have diagrammed it for *Chlorella* and postulated an active pathogenesis relationship. We routinely looked at them microscopically, and I could see no evidence of it.

Dr. TSUCHIYA. Did you see a staph growing in the Knop's medium?

Dr. WARD. If you do not starve the cells and put them in Knop's medium, you will get what appears to be a little cell division. If you starve then quite a while, you will not get any. We also were interested in knowing whether or not active, "real hot" human pathogens would grow and survive in algal cultures. It has obviously practical implications as to whether or not you actually separate the manned component from the other components in the sealed system. We tried a number of different ones, and found that most of them, in fact, do not. We found two enteric passages that do reproduce in algal cultures, for what it's worth. We did a large number of experiments as to the kinds of organisms. We did not find them in the cultures we put them in to see if they would survive. One of the coauthors of part of this work did a number of experiments like this, putting in just about everything, and uniformly they would disappear. We only scratched on identification and tracing of the various compounds as they were being liberated and disappearing. The work has been terminated, and I do not know if we will start it again.

Dr. COX. Are these isolated initially from algae or are they mixed cultures?

Dr. WARD. This particular algae is a fresh water algae.

Dr. COX. I would predict that this is a case where you may be more interested in the organisms you did not find than those that you did. The point is that contamination is a relative thing. Even if you try to purify by, let us say, millipore filtration, there is a whole world of bacteria that will go through those filters.

Dr. WARD. I am aware of this, but we did not have any problem with this for the simple reason that the cultures were axenic to start with. We were growing them for the spin medium. They were clean to start with. Every tube was collected. If it had a contaminant, the whole thing was thrown out. They are not complicated in this way.

Dr. COX. They may never reach proportions that might permit you to find them, but they might be highly significant.

Dr. WARD. This is true, and I tried to point out in the beginning that we isolated only those occurring in large numbers.

Certainly if they are contaminated, they do not ever show up on the test medium we use. You only use a certain number. We use two or three different types of media for isolation, such as blood medium.

Dr. COX. You see only what you look for.

Dr. WARD. If we had taken everything we found, we would have had a vast area of bacteria.

Dr. DECICCO. I think this is very interesting that you keep finding the same organisms. The question arises why this complex medium, which develops after the algae you are growing, should support growth of a lot of organisms. I wonder whether it would be interesting to look at, for instance, antagonisms among the bacteria. *Pseudomonas acruginosa*, which you find among all the cultures you tested, normally inhibits all the bacteria. It is notorious for this. What you have may be *Pseudomonas aeruginosa.*

Dr. WARD. This is quite true. I do not know the reasons why I called it a competitive advantage. We do not really know the reasons that one can predominate when it is in mixed culture versus when it is a single contaminant, but they certainly act differently.

Dr. DECICCO. I am not sure of the experimental pro-

cedure. Was this with all the bacteria together? You tested utilization of the various compounds, or one at a time, plus algae?

Dr. WARD. One at a time.

Dr. DECICCO. One bacterium plus the algae?

Dr. WARD. Yes.

Dr. DECICCO. You had all the substrates available at the same time?

Dr. WARD. All substrates were available. We analyzed and quantitated without bacteria, and with that bacterium, plus algae—mixed culture and an uncontaminated culture. This is simply all it was.

The Case for the Multispecies Ecological System, with Special Reference to Succession and Stability

G. Dennis Cooke, Robert J. Beyers, and Eugene P. Odum
University of Georgia
Athens, Georgia

While in a space capsule, man is a member of an ecosystem and, consequently, will be affected by all that goes on in this ecosystem. The success of long-term space flight may well depend on our success in developing a stable and long-lived life-support system. This paper will discuss some properties of ecosystems which promote stability and longevity and will propose that life-support systems must be developed within the conceptual framework of the mature multispecies ecosystem.

Several types of life-support systems have been designed or suggested to handle the following aspects of astronaut metabolism: gas exchange, food production, waste disposal, and nutrient and water regeneration. Of those proposed, only the storage system, designed for short flights, has been successfully tested. For flights of long duration, only bioregenerative systems appear to be feasible. The two-species gas exchange and/or food production model has received much attention. These have been described as the unialgal-man gas exchanger (ref. 1) and the *Hydrogenomonas*–man life-support system (fer. 2). In addition, Oswald et al. (ref. 3) have discussed the feasibility of an algae-bacteria-mammal system. One other type of life-support system has been suggested. This is the multispecies climax ecosystem, proposed by H. T. Odum (ref. 4), which will be the topic of this paper.

There are at least two approaches to the development of life-support systems. One of these consists of testing and later assembling separate biological, chemical, and mechanical components. The second consists of allowing groups of species known to occur together to reassemble and reorganize in a new environment into an integrated, self-maintaining system; this we call an ecosystem. Nature operates by the second method. Man uses the first in constructing his machines. We submit that the first is ecologically unsound and will prove to be unsuccessful. The balance of this paper will present evidence to show that the multispecies approach will provide the greatest opportunity for developing a successful long-term life-support system.

An ecosystem is any assemblage of organisms and their abiotic environment that has the following characteristics (refs. 5 and 6): structural organization, interdependency of components, homeostasis and regulation (external, internal, or both), limits and thresholds, and a development toward a steady state with increasing adaptation with and control of the physical environment (succession). There are four components of an ecosystem: (1) abiotic substances (organic and inorganic), (2) producers (autotrophs), (3) consumers (phagotrophs), which feed on larger particles, and (4) decomposers (osmotrophs), which derive their support from smaller or molecular size particles. Both of these latter categories decompose organic material and release products usable by producers.

One of the most important attributes of ecosystems is the unidirectional flow of energy from green plants through food webs to consumers and decomposers. The amount of photosynthate stored in excess of daytime community respiration is termed "net community photosynthesis." At night, part or all of this net storage is consumed by community respiration. A continued excess of community photosynthesis over community respiration leads to an accumulation of biomass. Eventually this accumulation of biomass stops as limits of light-input utilization are reached, or nutrients become limiting, or some physical threshold (such as space requirements) is reached.

Ecosystems have structure: biomass, stratification of both living and nonliving substances,

and biochemical and species diversity. Depending on age and limitations of the physical environment, structure is more or less apparent in terms of the numbers of species. An ecosystem with many species per unit number of individuals may have a very complex food web as a result of niche (way of life) specialization by these species. Also, as the number of species increases, the number of homeostatic or regulatory mechanisms increases, and the organisms within and between the various trophic levels become more independent. There is increasing evidence to show that diverse ecosystems are also diverse biochemically (ref. 7).

The truly unique feature of ecosystems, however, is not structure or regulation, since these might be accomplished through external mechanical means, but the ability of ecosystems to develop, to come to a steady, self-maintaining, mature stage, often called a climax ecosystem. Some examples of mature systems adapted to particular physical factor regimes are temperate North American *Stipa-Biuteloua* perennial grassland, eastern deciduous oak-hickory forest, intertidal mangrove forest, tropical rain forest, and coral reef. For studies on mature natural ecosystems, see Golley et al. (ref. 8) and Odum and Odum (ref. 9). Oriental rice culture represents an agricultural system maintained by man which is more mature and stable than, for example, shifting row-crop tropical agriculture. The mature ecosystem tends to exhibit the maximum in structure and stability, within the limits imposed by the physical environment.

It is the mature ecosystem which we propose as the theoretical basis for the development of life-support systems. We believe that long-term stability, which is the result of the development of many homeostatic mechanisms through succession, must be the underlying concept in the development of a dependable life-support system. In other words, a multispecies system, with its associated high stability, has a far higher probability of survival than, for example, a two-species system.

A two-species system represents what we call "young nature." It has characteristics of early developmental stages of succession. A multispecies system, the result of a long developmental period, represents what we call "old nature" or a mature developmental stage (ref. 10).

A tabular model of succession (table I) has been prepared, with which we can compare the characteristics of old and young nature. Using some of the concepts of this model, we intend to compare two-species life-support systems and multispecies systems. We will show in greater detail some properties of ecosystems which demonstrate why we believe that a system with the characteristics of a mature ecosystem must be the basis of future life-support systems.

Not all of the ecosystem attributes of the model shown in table I are applicable, at present, to life-support systems. For example, no one envisions the possibility of man completing a life cycle in space. Other attributes are not well documented and need further research. Our remarks here will be confined primarily to attributes of ecosystem energetics and structure.

Many of the data which we will use to demonstrate the functional and structural events during succession have been obtained from the study of laboratory microcosms. These microecosystems are at least partially physically isolated from other ecosystems and in this respect are unnatural, since there is no export or import other than light and gas exchange with the atmosphere. However, the data obtained from these systems have particular applicability to the topic in question since a space capsule is a microecosystem.

The microcosm method has been described by Beyers (refs. 11 and 12). Materials from a natural ecosystem are brought into the laboratory and divided equally among a group of containers. Cross-seeding minimizes any possibility of divergence between microcosms. The systems are then placed on the desired regime of physico-chemical variables. Metabolism is measured by recording diurnal pH changes, and these data are translated into total CO_2 changes through the use of a graph depicting the relationship between microcosm pH and CO_2 changes. Microcosm biomass is determined by pouring an ecosystem into a tared weighing dish or through a tared millipore filter. The ma-

TABLE I.—*Model of Ecological Succession with Trends to be Expected in the Development of Ecosystems* [a]

	Ecosystem attributes	Developmental stages	Mature stages
Community energetics.	Gross production/community respiration	P/R ratio or one	Approaches one.
	Gross production/standing crop biomass	P/B ratio high	Low.
	Net community production (yield)	High	Low.
	Food chains	Linear, predominately grazing.	Web-like, predominately detritus.
Community structure.	Standing crop biomass & organic matter	Small	Large.
	Species diversity	Low	High.
	Biochemical diversity	Low	High.
	Stratification	Undeveloped	Well developed.
Life history.	Niche specialization	Broad	Narrow.
	Size of organism	Small	Large.
	Life cycles	Short, simple	Long, complex.
Nutrient cycling.	Free inorganic nutrients	Large	Small.
	Mineral cycles	Open	Closed.
	Nutrient exchange rate, organisms environment.	Rapid	Slow.
	Role animals in nutrient regeneration	Unimportant	Important.
Overall homeostasis.	Internal symbiosis (interdependence of organisms).	Low	High.
	Nutrient conservation	Poor	Good.
	Stability (resist external pertubation)	Poor	Good.
	Entropy	High	Low.
	Information	Low	High.

[a] Prepared by Eugene P. Odum.

terials are then dried and weighed. Similarly, total ecosystem chlorophyll is measured by filtering all or part of the system, reading the acetone extract at the appropriate wavelengths for the various pigments, and calculating the amount of chlorophyll according to Strickland and Parsons (ref. 13). These and other measurements are made at intervals during development or succession of the microcosm and, from these data, we have been able to show the course of some of the events of metabolic and structural succession.

In comparing the structure and function of old and young nature, we intend to emphasize these main points: (1) The two-species system represents young nature and has the advantage of a high rate of productivity per unit biomass, but with low stability. The multispecies system has a low photosynthesis-to-biomass ratio and must be large to support an astronaut, but it has the distinct advantage of multichannel stability. (2) The astronaut is part of a microecosystem, whether we are considering a two-species or a multispecies system, and therefore is part of the structure and function of the system. Depending on system stability, he will be more or less influenced by perturbations in its structure and function.

In figures 1 and 2, some data on succession in laboratory microcosms are plotted. In these experiments succession was initiated by innoculating material from a mature system into new medium. In young communities the rate of daytime photosynthesis exceeds that of night respiration, and biomass accumulates. Total or gross photosynthesis is high in the early stages. After about 70 days of succession, the ratio of daytime photosynthesis to night respiration ap-

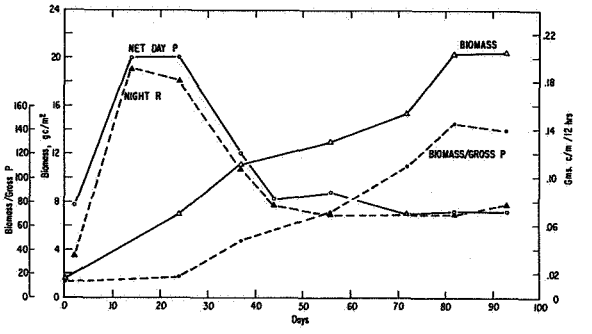

FIGURE 1.—Plots of net daytime photosynthesis, nighttime respiration, biomass and the ratio between biomass and gross or total photosynthesis against time in a microecosystem undergoing autotrophic succession. All data have been reduced to areal dimensions.

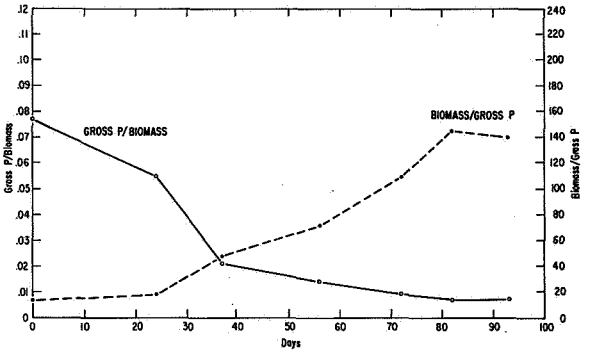

FIGURE 2.—Plots of the ratios of gross or total photosynthesis to biomass and vice versa against time in a microecosystem undergoing autotrophic succession. This figure illustrates the two types of efficiency outlined in the text.

proaches 1, and biomass reaches a stable value. At this point, the efficiency of the system is maximum, under a given set of environmental conditions, in that the highest level of biomass is maintained per unit of gross photosynthesis. Note also that the rates of respiration and photosynthesis are steady.

In the comparison of young and old nature, we are actually comparing two types of efficiency (fig. 2). In early developmental stages such as the two-species life-support system, the ratio of gross photosynthesis to biomass is very high—a small amount of structure is maintaining a high rate of photosynthesis. This is one type of efficiency, that which has been emphasized by proponents of the two-species system. If succession is allowed to proceed, whether by design or accident, the ratio drops. The trend in succession is to develop as large and diverse a structure, per unit of energy flow, as possible. Thus in early stages, the biomass-photosynthesis ratio is low; in a mature stage, the ratio is high. At climax a more complex structure, with a reduced waste of energy, allows the maintenance of the same biomass with a lower expenditure of energy or cost to the system (ref. 14). The more stable the system, both externally and internally, the less energy needed to maintain this biomass (ref. 15). In other words, as the system ages and develops structure, it becomes more efficient at maintaining that structure. This is the other type of efficiency, which we propose to be the basis of a stable life-support system.

The important point is that stability in these rates and ratios has been developed and will be maintained without external controls at maturity, while in young stages, stability must be constantly maintained through external controls. Mature microecosystems in the laboratory of Beyers have maintained themselves for years.

Another development during succession is the shift from an early planktonic-open water system to a detritus system in later stages. Recent studies (refs. 17–19) have shown that up to 90 percent or more of the metabolism of natural mature systems is in the detritus layer. The consequence of this in the use of a mature multispecies life-support system is that the astronaut must become a detritus-feeder, or a consumer of detritus-feeding organisms. This may, in fact, prove to be far more palatable than bacteria or algae since a great variety of vertebrates and invertebrates are detritus-feeders.

As communities develop, there is an increase in species diversity, and this has been assumed to contribute to stability (ref. 15). In a young ecosystem, there are a large number of unexploited ways of life, or niches. During succession, organisms from other ecosystems invade such structurally simple communities, or organisms that have been dormant or rare in the early stages become active and numerous. With time, the number of species per unit number of individuals, which we may call a species/number diversity index, increases. It is assumed on in-

complete evidence that an increase in the diversity index favors the establishment of homeostasis in terms of checks and balances. During the early stages of succession, there may be "blooms" of the invader populations, often at the expense of one or more established species. Such blooms create perturbations that may seriously upset the balance within systems as does a "cancer" growth in individuals. For instance, in the development of unialgal life-support systems, Miller and Ward (ref. 20) have remarked on the difficulty of preventing the establishment of large populations of grazing zooplankton in their cultures. In a mature ecosystem, with most or all niches filled, the probability of blooms, or even the successful invasion by a new species, is very low (ref. 21); that is, the system now possesses stability. For example, the probability of invasion by extraterrestrial species, or a bloom of a mutated form of some component species, in a climax system would be much less than in an unsaturated system.

To summarize, the presence of many species not only means a diversity of energy pathways, but also the presence of a great many regulatory and symbiotic relationships. We cannot envision a two-species mechanical-bioregenerative system with this sophistication of control.

Another important trend from young to mature ecosystems, which is a direct result of increasing diversity, is the increase in complexity of food webs. In young stages, the number of species is small and, therefore, the number of pathways of energy transfer between producers and consumers is limited. In the two-species life-support system, this pathway is linear. In older stages, a great many species have had an opportunity to become established and the food web becomes more complex—so complex, in fact, that few have been completely described for any large natural area. Figures 3 and 4, based on the data of Paine (ref. 16), illustrate food webs of a simple and a complex ecosystem. The importance of food-web complexity to ecosystem stability is very apparent here. The top consumer in the more complex food web has the choice of 10 prey, and most of these prey also have several food chains from which to graze; whereas in the less complex system, the number of interactions is much lower. This is analogous to the backup systems built into the circuitry of space vehicles. In the microecosystem, the investigator has some choice about the number of species in the system, at least initially. During succession, several of the original groups of species may become extinct. For example, truly planktonic species do not survive succession to the climax stage.

Several examples will illustrate our point about the stability of mature ecosystems. In a climax forest, the outbreak of a pest is rare (ref. 21), but in a corn field (an ecosystem much like the two-species life-support system) large numbers of pests are common. In the forest a system of checks and balances ensures that an increase in insect population density is automatically followed by an increase in predator density. No such system exists in the corn field, and the farmer has to resort to pesticides or mechanical devices. With increasing insecticide resistance, he must resort to more and more potent chemicals. In practice, a combination of biological and physical control is usually optimum from man's standpoint. Our point is that we should fully utilize all possible self-regulation so as not to create unnecessary artificial substitutes.

Beyers (ref. 22) has shown that the metabolism of a complex climax ecosystem is con-

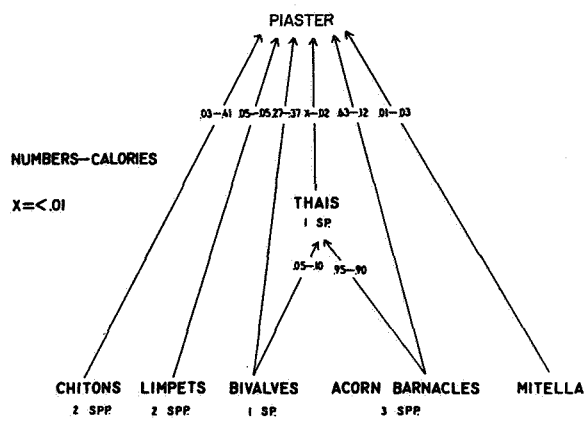

FIGURE 3.—The feeding relationships by numbers and calories of the Piaster-dominated food web at Mukkaw Bay, Washington. Piaster, N=1049; Thais=287, where N is the number of food items observed eaten by the predators. The specific composition of each predator's diet is given as a pair of fractions: numbers on the left, calories on the right (ref. 16).

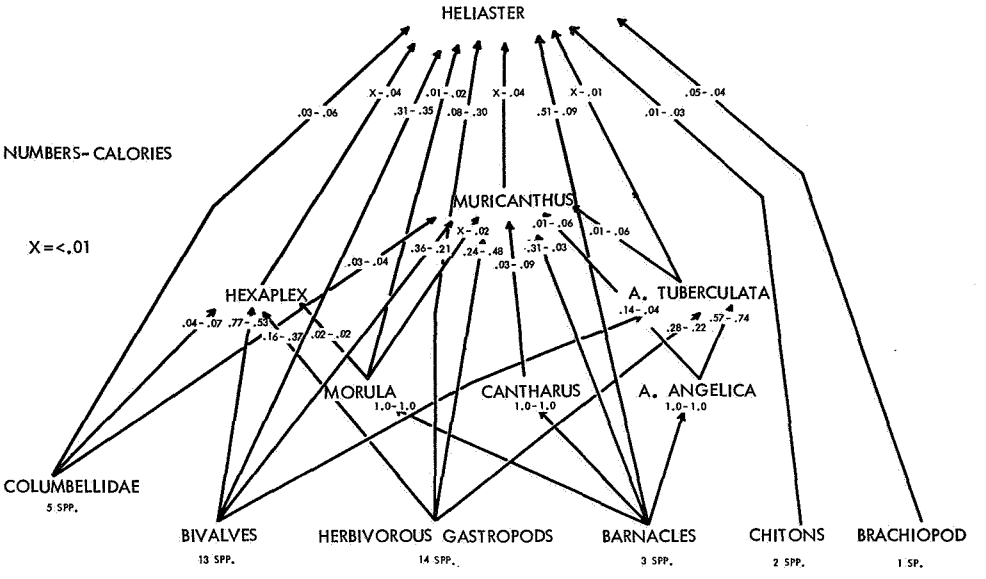

FIGURE 4.—The feeding relationships by numbers and calories of the Heliaster-dominated food web in the northern Gulf of California. Heliaster, N=2245; Muricanthus, N=113; Hexaplex, N=62; A. tuberculata, N=14; A. angelica, N=432; Morula, N=39; Cantharus, N=8. See figure 3 for further explanation (ref. 16).

siderably more independent of temperature than the metabolism of a simpler sewage community or a single organism. He postulated that the closer a living system approaches the integration of a balanced ecosystem, the less it is affected by temperature. This hypothesis may be expanded to state that the more complex the ecosystem, the less it will be affected by temperature extremes, adding stability to the system as a whole.

A mature ecosystem is also metabolically stable. Golueke and Oswald (ref. 23) have pointed out that the CO_2/O_2 exchange ratio of the plant used in setting up a biological gas exchanger must match that of the crew, since a very slight mismatch between human RQ and plant AQ would lead to the accumulation or loss of a certain fraction of the human oxygen demand and carbon-dioxide output per day. In the multispecies system, the RQ's of several kinds of heterotrophs would balance the AQ's of the several autotrophs so that a temporary imbalance with one species would be compensated for by other species.

Additional evidence of the increased stability for a multispecies system is shown by the data presented in figure 5. A climax microecosystem was irradiated with 10^6 rad in an acute dose. With the exception of the loss of one species (an ostracod), there was no visible effect on the system. However, when the system was used to initiate a new autotrophic succession, the results of radiation became apparent. As can be seen in figure 5, the rate of growth of the system was

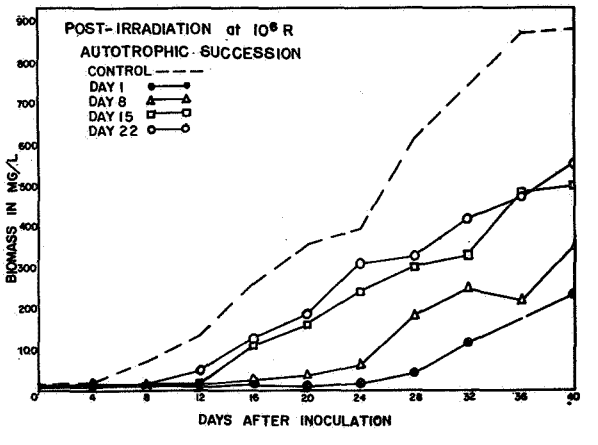

FIGURE 5.—Course of biomass increase with time in an autotrophic succession in a laboratory microecosystem irradiated at 10^6 rad. Successions were initiated by inoculating samples of the irradiated mature microecosystem at 1, 8, 15, and 22 days after irradiation. Control curve is from nonirradiated microecosystems.

decreased in comparison to the nonirradiated controls. However, this effect decreased with time, indicating the system's capacity for self-repair. The curve for each innoculation, made at weekly intervals after irradiation, shows progressive recovery. The greater the time after irradiation, the closer the curve approaches that of the controls. The principal primary producer and the organism accounting for the maximum biomass in this microcosm was a *Chlorella*. It is interesting to note that Posner and Sparrow (ref. 24) found that 90 percent of their pure culture *Chlorella* died after a dose of 23 000 rad. Our *Chlorella* showed no effects of radiation until they were irradiated at 2 000 000 rad and then took 40 days to die. These results may indicate that the system confers some radiation protection to its member organisms.

The interdependency of components in a climax microecosystem was clearly illustrated in a recent investigation in our laboratory by R. Gorden. Gorden has shown that a bacterium also present in the climax is an important source of thiamine, a requirement for the growth of the *Chlorella*. Close symbiosis between pairs of taxonomically unrelated species is an outstanding characteristic of the most successful natural communities, for example, lichen growths in the arctic or coral reefs in the tropics.

Although little information is presently available, it appears that during succession both the variety and amount of biochemicals increase (ref. 7). Many of these extrametabolites apparently have the properties of inhibitors or of growth promoters (ref. 25). These substances then are environmental hormones and act as regulators (ref. 7). Another biochemical change during succession is the increase in the quantity of accessory photosynthetic pigments, thus affording the ecosystem with more complete utilization of light as well as a complement of more stable pigments. The increased ability of mature systems to regulate themselves by internal chemical feedback means that less outside energy need be applied by man to achieve stability.

Beyers (refs. 11 and 26) has shown that there is a general pattern in the metabolism of aquatic ecosystems correlated with the onset of light and dark. The maximum metabolism occurs in the first half of the day or night period. The implications of this pattern for multispecies life-support systems have been discussed elsewhere (ref. 27). It must be admitted that there is the possibility of deleterious effects on an astronaut by this periodic reduction of photosynthesis and respiration. However, it does seem that the intensity of this phenomena decreases as the species diversity increases (ref. 11), adding another bit of evidence for the use of complex systems to support man in space.

Our main point in this discussion of ecosystem structure and function is high diversity and high stability in mature systems, low diversity and low stability in early stages. The more mature system has a builtin set of checks and balances which prevents internal disturbances and buffers the system against most external disturbances. Of course, no system is immune to severe perturbations and, in fact, the limits of ecosystem stability are strongly related to the stability of the physical environment (ref. 28).

There are certain distinct advantages to young nature, when viewed as life-support systems. These advantages are primarily energetic. That is, young ecosystems have high productivity rates per unit biomass, which means that they are more efficient gas exchangers in terms of O_2 produced or CO_2 absorbed per unit of biomass. As we have pointed out, however, there are serious disadvantages to young systems as well. It should now be apparent that, in the development of a life-support ecosystem for man, we must first select for system stability and longevity and then turn to the development of maximum productivity per unit biomass consonant with this stability. We cannot, as has been suggested by Miller and Ward (ref. 20), simply select organisms as needed on the basis of certain desirable functional characteristics and hope to integrate them successfully into a system. This attitude implies that the addition of new species will have no effect on resident species and this, of course, is ecologically unsound. It should also be apparent that homeostatic mechanisms of ecosystems are far more sophisticated and reliable than their

mechanical counterparts. As H. T. Odum (ref. 4) has pointed out, man has yet to develop the miniaturization of circuitry that is found in ecosystems.

The area of a multispecies life-support ecosystem capable of supporting an astronaut has been estimated at 2 acres (ref. 4). This estimate is based on an expenditure of all but about 2 percent of the photosynthetic production on respiratory requirements of other components of the system. Obviously, practicality dictates some compromise between two-species mechanical systems and the multispecies system which depends on natural self-regulation.

To date, the major emphasis in the development of life-support systems has been on single components. These data are valuable and such work should be supported in the future. However, we believe, based on our knowledge of the properties of ecosystems, that future work must stress the development of a multispecies system.

Since such a system will necessarily be larger than proposed two-species systems, we need to determine which processes can be satisfactorily supplemented or replaced by mechanical or chemical devices. For example, the reduction of fecal material to small particles might best be handled by some mechanical method, thus eliminating the need for populations of consumers which ordinarily would fill this role. In other words, we may be able to reduce the predicted size of a multispecies life-support ecosystem without reducing the built-in stability of the system.

Finally, it is clear that a great deal of work must be directed toward an analysis of properties of ecosystems before we attempt to devise a multispecies life-support system. Part of the controversy regarding the simple vs complex system may be resolved by a set of experiments. Laboratory microecosystems ranging in complexity from a single alga and consumer to a highly complex system containing several representatives of each trophic level could be cultured under identical conditions. The climax systems and successional stages could be tested for metabolic and species stability under various stresses. Such stresses could include thermal manipulations, ionizing radiation, invasion by foreign species, and mechanical and photoperiodic stresses. We propose to perform such experiments in the future.

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COMMENTS

Dr. WARD. I would say that your suggestion is exactly that made previously by Jack Myers and later by myself and Dr. Miller. That is, a one-by-one selection of species and a study of their interactions where one knows what one has. Then build it up until you get your complexity from which you get your stability, rather than starting with a bucket and never knowing exactly what you have, where it is going, or what happens to it.

This last experiment you suggested is basically a rewording of the one-by-one selection to study what one can get away with towards life-support. I do not see that your suggestion is really divergent from what we have already suggested.

Dr. COOKE. This is not precisely what I am saying. Let me go back a way in the history of ecologic research and thought.

Most of the research in ecology has been done on simple single species or a pair of species. For example, one goes into the forest and studies the maple trees and some other fellow comes in and studies oak trees, or a variety of things. Then they hope to describe the ecosystem from what they have learned about each of these single species. As it turns out, the whole is a little greater than the sum of the parts. Consequently, you have to look at the whole system, as well.

We know from experience that, if you have an ecosystem which you have learned something about and have some data on, and then add a new species, you are going to have a new succession start, whether it be small or large. Our opinion is that, if we are going to try to do this kind of thing, let us put them together and learn how they interact. If one wants to learn specific processes in single organisms, fine. Perhaps we can get some physiologist to attack that, as well. It is the difference in the concept.

Dr. REPASKE. I believe there is a fundamental difference in principle between your ecosystem and what will occur in the space capsule. In your ecosystem, it is immaterial which species gains ascendency and which goes down.

In the capsule, we certainly are interested in one species maintaining itself, namely, the man. If you, in your ecosystem, would be interested in one of your flagellates or in one of your organisms, and had to do everything in order to have this maintain itself, then you would have an analogous system that we have in the capsule.

But, if you are just concerned about some life existing in some balance, and it is immaterial as to which species predominates, then I believe it is an entirely different system.

Dr. COOKE. I see your point. We, too, are interested in the astronaut surviving. That is our main point.

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If it takes a big system to support him, then it will have to be big. If he is one of many components in there, that is just the way you will have to look at it. We cannot sacrifice stability. This is the important thing.

Dr. REPASKE. I believe your approach is wrong, because, if you would propose your microecosystems from the standpoint of having one species and arbitrarily try to maintain this species in some constant number and let your other fluctuations occur as they do occur, then you have something analogous. If you let everything fluctuate randomly at will, you are comparing one system that has no basis for comparison with the other.

Dr. COOKE. I agree that these two systems are completely unalike. In other words, if we had a microecosystem in which we intended to maintain a fish in it, as a top consumer, we would have to make some efforts to see to it that everything that is needed for that fish is in there. This would be a pretty complex system in comparison to what we have. We have no vertebrates in it.

Dr. KRAUSS. I only wanted to remark that I believe there is nothing that you have said about the stability of ecosystems with which would be debated by any of us who have anything to do with ecology. But, we are dealing with a rather special situation here of a man in a space capsule. It is a pretty unstable situation, regardless of what the life-support situation is. But, because of trading off stability of an ecosystem to engineering, we are able to maintain just one species, as a matter of fact, for a reasonably long time.

We have had one up for the last 5 or 6 days, for instance. But you see, by introducing one or more microorganismal systems, we are, in fact, moving away from that single species to at least two or three, or something of this sort. The whole question in deciding about long-term life-support, I believe, is going to revolve about where you can trade off stability for engineering. A stable system is a tremendously expensive system from the point of view of space, weight, and energy consumption. A much more efficient system is a much more compact, compressed system, admittedly, with the dangers of instability. In this particular kind of situation, I think one has to invoke these basic principles of ecology with great caution. We are doing something which, in itself, is unstable, and is being maintained for a fairly limited period of time. With all of the resources of stability of the planet behind, it is compressed into an extremely efficient, functioning system for a relatively brief period.

Even a thousand days is a relatively brief period, but this thing comes out of a very broad-based stable ecology on the planet of which man is a part, an increasingly less stable part (as you well know), but still a part.

Dr. COOKE. Are you using the word "efficiency" a little differently than we are? We are talking about the efficiency of high production and small size.

Dr. KRAUSS. We have a specific mission here, you see. That is to get a man up for enough time to do whatever observational work he has to do and to get him back with the minimum of weight and volume to go with him during that period of time. Of course, we sacrifice much of the very sound and indisputable arguments of ecology in order to bring this about.

Dr. KOK. I still think there is no sacrifice whatsoever in the two-component system. The second point is right in your graphs. By including even three more components, your efficiency goes down with such dramatic numbers that in the very first curve you showed a factor of a 1000 in it. Not only is it a fallacy in stability conception, but also the efficiency is inhibited.

Dr. COOKE. I do not agree with saying there is a fallacy in stability. Would you rather ask whether an astronaut will come back or not?

All we are saying is that you have to invest some stability in the system, and engineering has not shown itself to be quite that good. How can you beat something that evolved for millions of years? That is pretty efficient engineering. You are dependent upon an ecosystem that is stable.

Dr. JENKINS. That is just the reverse. We have monocultures all over the world. Our whole agriculture is based on single species. Nobody tries to grow rice, peanuts and orange trees in the same soil, and we are talking about a system now where we have a few cubic meters, to support 8 or 10 men.

While your principles are correct, what relationship does it have to the spacecraft or even carrying it out, if you would extend this, to a lunar colony or to a Martian colony many years in the future? Would you ever get past the monoculture that we have on Earth?

Dr. COOKE. Your point is well made that obviously our agriculture is essentially a monoculture. Of course, we are sacrificing a lot of stability in our own largest earthly environment as a result of this monoculture. This is something else that ecologists are going to have to speak up about. We are getting more and more unstable. The temperature of the Earth is rising because our monoculture is attempting to replace fossil energy for solar energy through our own photosynthesis in our biosphere.

Dr. FOSTER. To put it more crudely, we want to bring them back in either case, but from an engineering viewpoint you are either going to scrub the trip completely in favor of stability or you are going for a thousand days, taking along with you as your major purpose, the best controller that has been so far invented, and that is man.

With certain instrumental aids, you have a man there who is operational and can control far better in a limited ecology than any other stable control that you can get.

Would you say that if there were going to be 10 or 20 species, this would be a reasonable estimate or would it be something else?

Dr. COOKE. I do not know.

Dr. TISCHER. If you were to set up a bacterial experiment at two levels, which is about as few as you can use to gain any information, you would have 2^{10} power experiments which would mean for one replication you would have to do a thousand determinations. If you double that, you have 2^{20} or a million determination, roughly. And so on and so on. It seems to me that added to the more simple cost factors involved is the cost of experimentation.

Dr. COOKE. I agree that it is an exremely complex problem. I can think of a lot of experiments, for example, that could easily answer some of your questions. It would be very nice, for example, to contain a small mammal, a rat or a mouse, or some other type organism, and attempt to build an isolated self-maintaining ecosystem. Such attempts have been made.

Dr. WARD. Do you consider Oswald's and Golneke's microecosystem as having considerably more stability than the other algal cultures, such as Dr. Krauss' which runs for weeks at a time? They had species diversity, in fact, quite a few. They had a pond of algae with associated bacteria that come out of an oxidation pond, plus the ones that inherently inhabit the gut of a mouse.

I do not see that any evidence to the fact that that particular system had turned out to be really any more stable than Jack Myers' balanced system, where he worked for several years with mice in algae cultures, or the stability of Dr. Krauss' algae cultures.

Dr. COOKE. We have yet to be impressed with a stable two-species ecosystem. I have not seen any evidence of it yet, if we want to call this an ecosystem. I have not seen a graph or paper that shows me a stable two-species life-support system that lasts more than a few hours to a few days.

Dr. KOK. Spaceflight is not interested in the ecosystem.

Dr. COOKE. A two-species system is an ecosystem. Have you shown me a system of life-support that has been stable for a long period of time?

Dr. DECICCO. I think we are confusing a completely closed system with a partially or mainly closed system. I do not recall anyone ever saying we could have a two-species, completely closed ecosystem. I think what we are trying to get at is as close to this as possible. We are exploring how complete a system we can get with two species and compare this to how complete a system we could get perhaps with more species. Then we look at the engineering problems involved in both and try to determine which would be the most advantageous.

Dr. REPASKE. Do we really want an ecosystem or just a supplement to maintain a man? If the spacecraft were large enough, he could take corned beef sandwiches and oxygen, and we won't be concerned about an ecosystem at all. Because he probably cannot take enough tanks of oxygen and cannot take an adequate store of food, we simply want to supplement that which we cannot carry. I wonder if this necessarily involves us in an ecosystem or just a continuous supply of food and some exchange of atmosphere to maintain him?

Dr. COOKE. I think one shifts from the other, depending upon how long this voyage is going to be. I suppose the storage system could support a man maybe 30 or even 60 days. With complete storage of all the requirements in terms of tanks of gas and corned beef sandwiches, one gets past this point, and, sooner or later, you will have to have a more and more complete system for him.

Dr. JENKINS. The system has been designed for 700 days, four men, complete storage of food and oxygen. This is proposed for a Martian fly-by. However, when you get into 8 or 10 men and 1000 days, and some of these men landing on Mars, then it looks like the storage is getting out of hand. So a partial system of regeneration, at least for a partial regeneration of oxygen and water utilization, is needed. Actually, when you send up a man, you are sending up quite an ecosystem. There are many species of bacteria. In fact, if you didn't have a series of bacteria, the man probably would not have proper digestion.

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CONFERENCE CONCLUSION

Concluding Remarks

Dr. Cox. There will now be a summary discussion with conclusions and recommendations coming from all of the participants.

I have only been reading in this area for a very short time, but the hydrogen bacteria that I have read about are quite interesting. Actually, a wide variety of bacteria can catalyze the oxidation of hydrogen. These include sulphate reducers, *Clostridia*, *Streptomyces*, micrococci, *Pseudomonads*, and others. If a *Pseudomonad* can produce hydrogenase and also utilize CO_2 as a sole source of carbon autotrophically, then it has become customary to place it in a special genus called *Hydrogenomonas*. It is also clear, however, that some bacteria described in the literature, as *Hydrogenomonas*, are not known to be strict *Pseudomonads*. If they are not known to be strict *Pseudomonads*, what are they? The autotrophic hydrogen bacteria that we hear about, and apparently those discussed in this conference, also seem to grow as well, if not better, on organic substrates.

I do not know of any bacteria, autotrophic or otherwise, that have shown to be obligate hydrogen fixers. Therefore, it seems to me that the use of the genus *Hydrogenomonas* may be misleading; it may be confusing. I believe it probably should be abandoned. It might be very much better to look upon heterotrophic bacteria, all as being potentially capable of producing hydrogenase, regardless of the physiologic group that they belong to—autotrophic bacteria or heterotrophic bacteria—grown under autotrophic conditions.

I mention this because when someone says *Hydrogenomonas*, they should know something about the organisms that they are working with. If someone says he is working with a *Pseudomonad*, we immediately know certain things about it. If we want to do further research on this organism, we have a vast body of knowledge published that we could go to and get a rather good baseline for our study. I am really talking about saving time. My fear in sitting here again as an interested bystander is that I wonder about many of the *Hydrogenomonas* cultures that have been reported. I wonder what they are? I am even more confused because I cannot find, in the published literature, a description of the *Hydrogenomonas* specie *eutropha*, which apparently most of you are using.

This does not detract from the use of the organism at all. But, I would make a plea that, if it is that good, someone should write it up so that I can read about it.

Dr. Repaske. Biotechnology and Bioengineering has published some of the characteristics quite recently.

Dr. Cox. Do all of you think the *Hydrogenomonas* you are using are *Pseudomonads?*

Dr. DeCicco. Most of them are *Pseudomonads.*

Dr. McFadden. Duderoff has long been concerned about this very point.

Dr. DeCicco. When Duderoff says *Pseudomonas*, I know what he is talking about.

Dr. Johnson. He has a graduate student who just completed a dissertation on the taxonomy of *Hydrogenomonas*. I assume they will be publishing this at any time.

Dr. Cox. The plea I am making, really out of compassion for the organism, is to please pay some attention to the basic physiologic and anatomic structure of the organism, rather than merely assuming that when you get a culture and call it *Hydrogenomonas*, you know it produces hydrogenase and will grow autotrophically. This is all you care about. I can see where this kind of thinking can lead to trouble in time to come. The immediate question, it would seem to me, is in which characteristics of a microorganism is the space effort and NASA interested? What characteristics should an organism have? If a culture with such characteristics exists, and I presume it does in *H. eutropha*, then it would seem that a very extensive basic physiologic, metabolic, genetic, and perhaps even fine structure study should be performed quickly—studies like some of the

ones that we have heard here the last 2 days, but even more extensively, if no such culture exists. Attempts should be made to isolate one using appropriate enrichment techniques. I do not know how many people have attempted to isolate organisms from nature using enrichment techniques designed to select characteristics in which you are interested. I think I would like to organize the conclusions, recommendations, and discussions here by saying that we should probably pay attention to the problems that exist.

In other words, what are the problems and what progress has been made towards their solution?

Yesterday morning I heard Dr. Saunders mention a list of problems areas. I wonder if I could begin by asking him to repeat those slowly?

Dr. SAUNDERS. In following the proceedings of yesterday and today, I believe that a good deal of information was presented on many of the problems. However, I am not sure that all of these have been answered.

The first one dealt specifically with the *Hydrogenomonas*-chemosynthesis system. One of the problems was: Is there an inherent danger of damage to the enzyme system hydrogenase by virtue of the molecular oxygen in the medium?

Dr. Repaske, you discussed hydrogenase and I think Dr. Bongers mentioned something about the effect of excess oxygen on hydrogenase activity.

Have we satisfied this particular problem? Is there anything more that we can think about with that particular system?

As Dr. Cox mentioned, we have an organism with a hydrogenase system. It could be one of the organisms we might use in the system we are discussing.

Dr. REPASKE. If you are asking for answers, we have grown *H. eutropha* with as much as 25 or 30 percent oxygen and growth still occurs.

Quantitatively, I cannot recall whether growth is as rapid or not. As I recall, it is virtually the same, so at least to 25 percent or to 30 percent oxygen, one could assume that there is no effect.

Dr. SAUNDERS. It would not be a toxic effect of oxygen, per se?

Dr. REPASKE. Not in an autotrophic medium, at least.

Dr. BONGERS. I am not too convinced that we have only one hydrogenase, but that is beside the point.

There probably is an effect of oxygen on hydrogenase in that, if the oxygen concentration is high, the amount of hydrogenase formed in cells is low. While, if the oxygen concentration is low, the amount of hydrogenase formed is higher.

However, if you transfer from high oxygen concentration to low oxygen concentration, there does not seem to be an inhibition. I would not see that there is any danger of oxygen on hydrogenase research. There is no experimental indication for that.

Dr. SAUNDERS. If there is an inhibition, it is a temporary or transient and a reversible type. You are satisfied then that we do not have much of a problem with this?

Dr. DECICCO. *Hydrogenomonas eutropha* has a constitute of hydrogenase, at least the kind we have mentioned. There may be more than one. The one we have been measuring is constitutive and this is regardless of what you grow it on, autotrophically or heterotrophically. We should keep in mind that this organism is a soil or water organism. It normally grows under an atmosphere of 20 percent oxygen and we never, to the best of my knowledge, under normal conditions, grow it in an atmosphere containing more than 20 percent oxygen. Normally, it is down to 10 or 15 percent. From this point of view, again going back to its ecology, it has been around for probably a few thousand years or more, growing under 20 percent oxygen and still has hydrogenase.

Dr. MCFADDEN. Do any of you working with *H. eutropha* see this in *H. facilis*?

We have looked at this with both *H. eutropha* and *H. facilis*. We tried to select by natural means and by artificial nutrients, obligate heterotrophs. Until recently, we had never been able to do so. This is using replicating and every other method.

We are checking every cell present in a large

population. Under natural means, we have never obtained an obligate heterotroph.

Recently, we have obtained a couple of organisms that seemed to be obligate heterotrophs, but only under certain conditions. I think this would be a very rare thing.

Dr. Cox. Have any of you seen obligate autotrophs in any of your cultures?

Dr. DeCicco. No. It has never been reported, to my knowledge.

Dr. Cox. What about *Nitrosomonas?*

Dr. Bongers. It has no hydrogenase.

Dr. DeCicco. They are nitrogen, you know.

Dr. Cox. Are you sure?

Dr. McFadden. Yes.

Dr. DeCicco. I am not sure about the hydrogenase.

Dr. Tischer. I wonder if the fifth answer of the high temperature would not argue for the fact that there is not one, but perhaps several dehydrogenases possible in this series of organisms, since, otherwise, how could this thing exist comfortably at 50 to 55°C?

Dr. Repaske. One step in my purification is to heat the enzyme preparation to 50 or 55°C. for 5 minutes. I get rid of much protein and virtually no hydrogenase, so the hydrogenase outside of the cell is stable to 50°C.

Dr. Tischer. We are talking about growing ours for weeks.

Dr. Repaske. I cannot argue what happens inside a cell versus what happens outside. It may be more stable inside the cell. Enzymes usually are more stable intracellularly than extracellularly; in an extract, it is stable at least 5 minutes at 50° C.

Dr. Tischer. I did not make any statement as an element of proof, but merely as a suggestion. This might conceivably turn out to be an element of proof at some time that there is some real difference between at least two or among several dehydrogenases coming from obstensibly different organisms.

Dr. Saunders. It is possible you are working with the same enzyme system, but that the features expressed belong to the iso-enzymes of the hydrogenase?

Dr. Tischer. It could be many things, of course, but I am looking at it in a gross sense. *H. eutropha* grows best at 50 to 55°C. There is obviously some difference. The question then is: Is this in the enzyme systems or is it not? I am really raising a question rather than answering one.

Dr. Repaske. The cytochrome oxidases that I mentioned yesterday, heated to 40°C, is completely inactivated. It seems that if one looks for differential susceptibilities to heat, the oxidase is more susceptible than is the hydrogenase. Still, this does not prove anything.

Dr. Tischer. What we might do is attempt to repeat the experiments you have done with the *H. eutropha* and see if the hydrogenase reacts the same, except, say, 10 or 15 degrees higher.

Dr. DeCicco. I might add that studies of this type have been performed with other enzymes and other organisms.

High temperature mutants have been selected and the most heat-sensitive enzyme has been isolated. It has been known also to be extracellular and more heat resistant. This has been correlated with differences in the amino acid sequence, etc. It is quite possible your organism has a somewhat changed hydrogenase that makes it more stable.

Dr. Tischer. It seems almost incumbent upon us to do this particular piece of work which we might not have undertaken otherwise.

Dr. Saunders. The second problem that I mentioned was the relationship between *Hydrogenomonas* and any bacteriophage. Dr. DeCicco presented information on that. However, in categorizing a relationship between the bacteriophage and *Hydrogenomonas*, it was brought out quite clearly yesterday that a contamination occurs by organisms in human urine, as well as possibility of any effects from carbonaceous organic-type products that might result in the medium.

The genetic stability is still a problem that really needs looking at very carefully. At the same time, we lump under the genetic stability of the *Hydrogenomonas* organism the factor brought out yesterday and today on contamination by either the bacteria themselves or other carbonaceous organic-type materials.

Dr. DeCicco. I certainly think that the over-

all stability of the organism, genetic or otherwise, must be studied to some further extent. With regard to the phage, we still should know or make an attempt to find out whether these things are lysogenic. If they are, you may be carrying them right along with you in a spacecraft. When you get hit with irradiation in outer space, mainly lysogenic culture, all of a sudden you have a bit of a problem. So I would say that this certainly should be looked at.

Dr. SAUNDERS. We found a lead in the 3-day biosatellite experiment with *Salmonella-typhimeurium*. In running these experiments through the rigors and paces of the flight profile in preflight tests, for hardware and systems tests, the combination of vibration and 1200 rad of gamma radiation shows a tendency toward a synergistic effect, in that there were more viruses following the 3-day simulated flight than there were originally.

What you are saying is that, if there is a phage in this organism and if it flies and encounters a radiation dose (we are hoping there will be adequate shielding), it might affect the organism. This is an important problem.

Dr. DECICCO. First we can look at the phage we have. Then we can look at some of the natural cultures we have been using all along.

With respect to the contamination problem, I wonder if it would not be expedient for all of us to collect our contaminants for awhile and maybe have some place where we can get them analyzed and see if we have any sort of correlation? We might then be able to determine whether with our cultures we pick up all sorts of contaminants or whether we, too, are selected for specific types. This could be extremely valuable, because, for one thing, I have been thinking of ways of controlling our culture by an antibiotic treatment or something like this, if contamination should occur during flight.

Since these organisms are quite resistant to both penicillin and streptomycin, either or both of these could be used for controls during flight. If we were checking our contaminants and found out how they work with respect to the sensitivity to these antibiotics and some other factors, this could be helpful.

Dr. Cox. I wondered if you established any relationship between your phages and the phages that are known for other *Pseudomonads*? I think this would be interesting.

Dr. DECICCO. No, I have not. One thing, which should be done in answer to a previous question you raised, is to take a hydrogen bacteria, throw out its hydrogenase and classify it. We had someone do this some years ago. It came out to be most closely related to a *Pseudomonas ovalis* from Bergey's Manual.

Tobacco mosaic is another case in point. The melting-temperature studies everybody did on DNA from *Hydrogenomonas* fall right in with it.

Dr. Cox. *Pseudomonas ovalis* is very easily confused with an organism now called *Pseuomali*, which apparently is a human pathogen.

Dr. DECICCO. I might also add that these resemble one another. They both have a reddish-brown pigment which is characteristic of a lot of *Hydrogenomonads*. I had a student run nutritional studies with *H. facilis* and *H. eutropha*. They are very different, even though she classified these by nutritional requirements.

Dr. Cox. I have a good friend whose judgment I respect and I offered to bet with him a couple of weeks ago that he could not go out into the soil and enrich for an organism which he might call *Aerobacter* and get hydrogen fixation. He immediately took me up on it. He said he would like to get money like this any time. I am not so sure this would be difficult. I don't know.

Dr. DECICCO. After *H. facilis* was tenatively suggested as being related to *Pseudomonas ovalis*, we took these organisms and attempted to grow them autotrophically. This was not successful. *H. ovalis* does not have a hydrogenase. It also is able to fix small amounts of CO_2 while metabolizing under hydrogen, but cannot grow autotrophically, at least under the same conditions we used.

Dr. Cox. Are these cultures which you isolated or stock?

Dr. DECICCO. These were obtained from the ATCC.

Dr. MCFADDEN. In this context there is one interesting property of *H. ovalis* which I recall from some work done in England 2 or 3 years

ago. It does have the complete autotrophic machinery for CO_2 fixation. It can grow at least some strains as the sole carbons are sought, oxidize others forming to CO_2, gets energy and electrons from this process and reassimulates the CO_2. So I find your comments quite interesting.

Dr. DeCicco. There is probably some small difference.

Dr. Saunders. I would like to move on to the next problem I raised. If I understood Dr. Calloway yesterday, she said that she was handing us a low blow from the standpoint of the nutritional qualities of the dried product.

Dr. Schwartz. I believe that, basically, we have to take Dr. Calloway's final comment seriously, that the one organism approach is going to be mighty difficult. Certainly I would like to repeat at this time a corrolary comment I made yesterday, and that is that I do not think it is realistic to assume that the organism per se is going to be usable. The biomass is surely going to have to be operated on in some way. Whether it is timely to do that when you still do not have your biomass defined is another matter from the strategy of how you develop this. It seems to me that the state of the art in moving biomass is fairly well developed.

All you have to do is to keep it in mind. You will have to sort out the various components and certainly, if you have a lipid fraction which seems to be as unsuitable or as unmetabolizable as these quick results indicate, you will have to do something to it to make it usable. It is clear that you cannot pile up huge amounts of unusable material from your biomass. You will have to do something with it in order to make your efficiency high enough.

I personally think that more than one organism is going to be necessary for another reason which has not been mentioned here. So far as we know, man does not function very well without a fair range of organisms in his gut. Somehow or other either we are going to wind up with a sterile man or one in which the gut organisms are stabilized and then come around and sterilize the medium in which you are growing your biomass. Or else, we will have, as Dr. Gustafsen suggested a few years ago, axenic astronauts, which might be a little difficult to achieve. I do not think you can avoid the clear indication that the gut flora are an important part of this story.

Dr. Bongers. I think we should point out here that these were preliminary answers as far as lipid utilization is concerned. Moreover, they received these samples only 3 weeks ago. A look at a different organism cultivated under quite different conditions (to me as someone who doesn't know anything about nutrition) was still astonishing that the protein, the lower level, was still digested in a similar fashion as the protein was in the normal organism. I think there will be a considerable problem in utilizing this polymer in which this work piles up. I think we are quite mistaken if we think that we can give quick answers to the difficult question.

Dr. Tsuchiya. This is the reason, actually, why we are interested in this entire business of mixed populations. They have not been able to use much more than 10 percent of algae and so forth, so far as man is concerned. I am not talking about rats or mice.

Dr. Schwartz. That certainly is true of yeast.

Dr. Tsuchiya. Therefore, I would be inclined to take seriously what Dr. Calloway will have to say about the upgrading of the protein.

Dr. Schwartz. I think there is another factor here and that is the balance of the diet. I think Dr. Calloway said she was going to go into a series of studies with very high protein. The organism tends to put out more protein than would normally be needed. What will happen when you load under other stresses with very high protein over a long period of time remains to be seen.

I think here expectation is that, in spite of Stephenson who lived on protein for a year and came out of that pretty well, you are going to have to find some way to readdress the balance. Also, there is a limit to how many calories you can get out of the fat, even if it is a good fat in the diet. Are you going to get more carbohydrates to balance this off?

Dr. Saunders. Dr. Johnson, could you comment on the nutritional approach being taken at Ames, whether they have done anything and obtained any information, other than this?

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Dr. JOHNSON. The studies are very preliminary because they did not have high *Hydrogenomonas*, although they contracted to get some.

They are trying feeding studies with *E. coli* and the last I heard the animals were still alive.

Dr. SAUNDERS. No breakdown of constituents?

Dr. JOHNSON. No. The whole cells are being fed.

Dr. TISCHER. I would like to make a comment based on some comments I had from Sir Hubert Wilkins. He described to a group some years ago the fact that pemmican, which certainly is somewhat like half-fat, is very well tolerated under Arctic conditions where temperatures are very low. Under a certain kind of stress condition, one can stay alive on pemmican whereas you cannot on Campbell's soup or something like that. The two just do not match under those circumstances. I am led to wonder whether the stresses of space travel may in any way have the same effect on nutritional capabilities of the astronaut, especially on long voyages. It could be that something which we feed here on the ground unsuccessfully would be tolerated very well. We do not have to presume lack of success in every unknown case. We can equally well presume a success. If this analog holds, then a fat which does not work too well on the ground might work fine under, say, considerable stress.

Dr. SAUNDERS. As I understand it, the astronauts that are flying are getting an Earth-type diet, except that it is dehydrated and must be reconstituted with water during space flight.

Do you know what the calorie count per day is? About 3000?

Dr. SCHWARTZ. Have they ever really consumed that, though?

My impression is that they have never really consumed the scheduled quantity of calories over a whole flight plan. Generally speaking, they have been under.

Dr. SAUNDERS. We do not have much information on the details.

Dr. TISCHER. I know one piece of information from Dave Simon's balloon trip—he ate nothing but candy bars in spite of the fact they gave him all sorts of other stuff to eat; he subsisted. This might conceivably mean something.

Dr. SAUNDERS. This still remains a problem. I believe most of you are agreed that we should wait for a final report on the critical analysis of her data, particularly the polymers that Dr. Bongers mentioned.

Dr. DECICCO. As far as protein goes, I do not really see where this is a problem. It might be an advantage instead. I do not think anyone ever suggested that these astronauts eat pure *Hydrogenomonas*. The diet will have to be supplemented with something else. Why can't the supplements be made to balance the protein and wind up with whatever type of carbohydrate-lipid balance you want?

Dr. SCHWARTZ. I think the problem here is that the major weight penalty in a normal diet is the carbohydrate. Really, if it came down to it, you could carry the essential amino acids for a very long trip without much trouble, if you were working at the minimum levels. In this case, you have protein in excess of basic nutritional requirement. The question is, can you just use it as caloric content without getting into trouble in other ways?

Dr. DECICCO. The carbohydrate is more expensive with regard to the weight.

Dr. SCHWARTZ. The normal diet is roughly 50 percent carbohydrate. If you start carrying that for a year, it gets to be kind of bulky.

Dr. WARD. Dr. Calloway's main difficulty has been lack of material to work with. I would suggest that this group might consider where she is going to get the material, if you want nutritional analyses performed.

Dr. SAUNDERS. How many of you are producers of adequate amounts of *Hydrogenomonas?*

Dr. REPASKE. I produced for my own consumption and then not enough.

Dr. TISCHER. We produced a little.

Dr. BONGERS. How much can we spare for Dr. Calloway?

Dr. FOSTER. We are getting 100 percent of our recovery, a pound every 2 months. This is recently. At lunch Dr. Calloway said she was not hurting so bad with the program she had active now, but if we gave her 100 pounds, she would start something else.

Dr. TISCHER. At this point, it might be well

to ask the question of whether we should not redirect some of our efforts towards producing organisms for Dr. Calloway's use as a very special part of the program?

Dr. SCHWARTZ. Don't you have to ask the question first: Is the composition of the biomass at this stage of development sufficiently reflected with where you think you will be when you work out some of the energetics, so it is worthwhile doing?

Mr. MCFADDEN. This is a very good point.

Clearly the polymer production varies considerably as a function of growth. This is just an example of one nutrient.

Dr. GOLDNER. If you are interested in just producing the polymer, disregarding the protein and other components of the cells, I think you could grow *Hydrogenomonas* heterotrophically very simply in mass culture and extract the polymer in its pure form. This would quickly produce voluminous amounts, without having the difficulty of autotrophic mass culture. We have been able to do this in our laboratory, by growing them on a very cheap heterotrophic medium and getting high yields of cells and optimizing for polymer production. As a corollary to this, I wonder if it is known whether the protein composition of the cells varies very much between heterotrophic and autotrophic growth. If not, would it pay to consider growing cells heterotrophically to at least get Dr. Calloway started along the way of long-term studies?

Dr. FOSTER. This was the information I had indirectly about Ames, that they were considering heterotrophic production.

Dr. JOHNSON. Both heterotrophically and autotrophically.

I am not directly associated with this myself, but my understanding is they are just worrying about themselves at the moment, but they might be prevailed upon.

Dr. TISCHER. We were under some misapprehension, because there were several things we mentioned last year that we have been waiting to see happen and apparently there has been a misunderstanding. For instance, I was sending these cultures to Dr. DeCicco and he told me he did not know why I was sending them to him and what he was going to do with them after he got them.

When I left the meeting last year, I was under the impression all of us would send these things to him.

Dr. DECICCO. I thought someone commented with me having the phage down there, maybe they better not send me the cuts.

Dr. TISCHER. Certainly our commitment was that if we could manage to do this in addition to what we were doing on our own part of the program, we would send all of the surplus to Dr. Calloway. Of course, we have not been in a position to send any significant amount. I think we could do this, if that is required, just as a separate function, on a batch basis. Not like Dr. Foster produces them, but by the methods we already use.

Dr. TSUCHIYA. I think the question raised about the quality of the protein varying under different growth conditions might enter into this.

Dr. FOSTER. This is the reason that we have been holding off, because we know the elementary composition changes drastically under different growth conditions. I had hoped that we would do what we have done 2 weeks ago. We have the path ahead of us clear as to how to get up to active growth under conditions that we project for the actual production continuance. Before we went into the 20-liter unit, we could turn out 100 pounds in 6 months, if we dropped everything else and did only that.

Dr. BONGERS. I personally think that nutrition is a bottleneck to your whole development. It may well be a good suggestion which Dr. Foster made, dropping everything else and producing the 100 pounds. You may well need it.

Dr. MILLER. What happens when you introduce the refinement and replenishing of the spent medium? This will introduce a further complication into your nutritional study.

Dr. BONGERS. There is one proof for this. I think all we sent to Dr. Calloway was about 3 or 4 pounds dry weight, but these were grown under autotrophic conditions under limitation of nitrogen. They were purposely made fat and

apparently her preliminary results showed the protein is not changed. Apparently, even under those conditions, the digestibility is just the same. It does not change that much.

Dr. TISCHER. Didn't we rather agree last year, or didn't Dr. Calloway say, she would rather receive it frozen? In other words, wet-frozen, rather than dried? That is what we planned to send her and have already sent her some.

Dr. SAUNDERS. As of 2 months ago, she changed her mind. It didn't matter.

Dr. BONGERS. I think she prefers lyophilized material.

Dr. FOSTER. As of this noon, she said frozen was fine.

Dr. TISCHER. Is it agreeable if we send her frozen?

Dr. SAUNDERS. If she says frozen is fine and prefers lyophilized, whichever way you can send it should do the job.

Dr. TISCHER. Lyophilization will cost us quite a bit of time and money. If we produced large quantities of cells, I would rather send them frozen, without doing any more than washing them and getting the excess water off them, rather than drying them by any method.

Dr. REPASKE. Could I suggest that we grow some cells heterotrophically with whatever complex medium we could decide on, have her get an amino acid analysis and run through a general analysis of this? If this organism happens to be the same in analysis as the autotroph, it would be a lot simpler to supply her large quantities of the heterotroph than it would be with the autotroph. If there is some fundamental difference between the two, then of course we would have to only use the autotrophic.

Dr. KRAUSS. It would be simpler to do it that way, but wouldn't it be a good exercise for you to incraese your autotrophic production here? I think you might learn something in the process.

Dr. JENKINS. In a magazine called Frontier, put out by the Illinois Institute of Technology, it says, "Approximately 6 tons of soft coal should give sufficient amounts of hydrogen and carbon dioxide for the production of one ton of hydrogen bacteria.

"Since the primary material requirements for producing hydrogen bacteria can be satisfied by a fertilizer manufacturer, a nominal integration of these is possible. Hydrogen would be generated for both processes, carbon dioxide and oxygen, by products of production and the fertilizer itself could be used to produce the hydrogen batceria."

I think it is important to consider the long-range value of *Hydrogenomonas* as one of the potential high protein foods in a future highly populated hungry world.

Dr. Humphries said that the Soviets had a symposium on *Hydrogenomonas* attended by 500 people. Their major interest was use of *Hydrogenomonas* for two reasons: (1) the basic studies and (2) the possibilities for food.

The food potential of *Hydrogenomonas* is an important thing. In our consideration of *Hydrogenomonas*, we are looking at it not only for space but as a spinoff for a potential food for the future.

Dr. SAUNDERS. This also applies to algal systems.

The State Department has asked us many times for information. I believe I referred them to Dr. Krauss once, about the possible spinoff from the algal research as a dietary supplement in Far Eastern nations.

In fact, the dietary supplement for solving some of the world's nutrition problems is one of the things that is keeping the oil business in the Middle East, because of the bacteria which they have isolated that can convert hydrocarbon to polypeptides as well as a certain amount of protein.

I mentioned a mutuality or commonality, for example, at the molecular level for energy collection and utilization, and so on, in the two systems that we were discussing these 2 days. Dr. Kok mentioned that he and Dr. Bongers conceived some sort of hybrid system at lunch time.

We discussed during the break the possibility of *Hydrogenomonas* being combined in the algal medium. Then Dr. Krauss gave us a beautiful illustration of a very expeditious system.

I wonder if the three of you and Dr. Ward might comment on it, in view of the nutrition problem that arose yesterday and the tendency

now to skim along and think at least of not a single organism system but maybe several.

Dr. KOK. Are the algae much better food?

Studies were done so long ago, who knows that? Who remembers that?

Dr. KRAUSS. It depends on what data you look at and who compiled them and what the source of the organism was. But we did some studies for NIH under good conditions which indicated that the algal material was very much like casein, as far as protein is concerned. Carbohydrates and fats were also reasonably satisfactory. I do not think any single food is in itself ideal. As far as the value of food products of *Hydrogenomonas* versus algae go, I do not think there is a major difference, unless it perhaps rests in the digestibility factor. I do not think there is much difference here.

We have talked in our laboratory a little bit about the possibility of trying to get a mixed culture going between some adapted *Hydrogenomonas* and *Chlorella* in the same vessel at the same time. But, I do not know very much about *Hydrogenomonas*. We are having enough trouble with the algae, so we never put them together. For those of you who know *Hydrogenomonas* and from what you have seen of our media and so forth, is there any reason why this wouldn't work?

Dr. BONGERS. You can grow them both together; I call this mutual acceptability for each other. I do not see any reason why it should not be possible. On the other hand, I do not see an advantage.

Dr. KOK. My feeling is tied to the speech of Dr. Jenkins. Do you in NASA want to do business or not? How fast does the thing have to be developed? How much technology has to be considered? Can you afford to keep on growing algae that cost—even if we keep on playing around freely in the laboratories? Maybe we will keep working until the thing is flying already without us.

Dr. JENKINS. The point I was presenting yesterday is that the time is getting late. Engineers at Langley Research Center, for example, are already considering chemosynthetic systems for space stations. Bioregenerative systems are not being considered by the engineers at the present time. When they talk to scientists working on bioregenerative systems, they hear about many biological problems with little statistically valid production data. Unless we develop a sound, reliable system the engineers will utilize a chemoregenerative system.

Dr. KRAUSS. From an engineering point of view, the engineers really do not like a man in the capsule.

Dr. WARD. This is appropriate. Are we progressing even conceivably at the rate we should to satisfy these engineering parameters? We have to be able, as biologists, to give them specifications at some fixed magic time in the future. I am afraid we are not advancing at that rate.

Dr. DECICCO. Obviously, I think the approach here is to find out from the engineers what they want to know.

Dr. WARD. They cannot tell you.

Dr. DECICCO. Someone has to decide. The problems you have been discussing are some of the things we have to decide and are involved in, but somewhere along the line we are going to have to find out what these people want to know, so we can supply the answers.

Dr. KOK. There should be engineers in this room, in other words.

Dr. JENKINS. The engineers need to know that a bioregenerative system will fit in a volume of a certain cubic size, with a specific weight, and specific power requirement, that it has a definite reliability that they can count on, and will produce under specific conditions definite quantities of oxygen, partial food supply for a definite period of time. When you furnish that information they will be happy because the chemoregenerative systems have problems of very high temperatures (500 to 600 degrees), production of toxic materials, and waste material.

Dr. KRAUSS. I agree with everything you have said. I think the point is that to get an apparatus that has that reliability and has the sophistication to go ahead and produce it for these long periods of time itself requires a higher level of engineering talent and effort—probably more effort and talent than has been available to the people working in the field. Technolog-

ically, the systems we are trying to create are far more difficult than creating a rocket engine or more difficult than the engineering of a capsule. Engineers seem to sympathize with the problems in developing these things, but do not quite sympathize with the very great problems of developing a biosystem.

Dr. SAUNDERS. We have this experience on record with the biosatellite.

The biologists are having their difficulties in getting the type of hardware that would keep that organism alive for the given mission. This, however, is a problem that is being resolved gradually. The communications factor between the biologist and engineer is being overcome gradually.

Dr. KOK. Ten years?

Dr. SAUNDERS. I do not think it will take that long, but it is a problem still with us, and I believe what Dr. Jenkins says is that, when you talk to an engineer about such a system being integrated into a spacecraft, he will ask you three questions—about weight, volume and power requirements, because those are constraints.

Dr. KRAUSS. How can you give him those, when you had only the very minimum experience with the kind of apparatus that you need? I think of our recyclostat for the algae, primitive as it is. I am not entirely satisfied with all its components. In looking at the turbidostat, assuming you are willing to settle for the medium washing through and out, it works also. But these are still relatively primitive devices. They are nowhere near the sort of intricate, involved self-sustaining devices that will be necessary, in order to give these figures a weight volume efficiency. It is expensive to build those things. It is much more expensive, I think, than anyone realizes. I have seen this with regard to the interest that the Navy had in life support for submarines. Somewhere along the line, there is a breakdown in understanding about what will really be required to get these to a functional state.

Dr. SAUNDERS. These are the things they would expect you to already have in almost a breadboard model.

Dr. KOK. It is very difficult to translate our machine into a space machine.

Dr. JENKINS. That is the responsibility of the engineers. The fundamental question is: Do you have a system that will work? Is it reliable enough for a period of time so that it is worthwhile carrying out tests in space under weightlessness conditions?

Dr. KOK. Take Dr. Krauss's system and work on it.

Dr. KRAUSS. That will be expensive. Dr. Bongers can help; I can help; and Dr. Foster can help. But to engineer a system like this would not be accomplished cheaply.

Dr. BONGERS. What you really want to have is a program to develop a system which might work under "zero" gravity conditions.

Dr. MILLER. The Air Force, at Brooks Air Force Base, has an overall objective for the development of a bioregenerative system. At least from a historical standpoint, the people in our group have been involved with the green-plant systems working mostly from an applied, and somewhat basic-applied standpoint.

We, at the present time, are activating a large two-man capacity algal photosynthetic exchange unit from which we hope to get logistics operations of just what is involved to get it operating, keep it operating, and maintain it, and what supply problems are involved. We are getting our backup information now, so that we can do the same type of thing Dr. Krauss is doing, the replenishment and recycling of spent medium. Also, we are working on waste reclamation reactors. We have two prototype activated sludge units, and we hope to tie those in, make up total algal units, and get this tied up to a chamber for a fairly extensive run some time in late 1968.

This is a development program which we have underway at the present time. There is also work going on on high plant systems, as well. We are all working on what was to be categorized as an unknown application right now, but high plant work is an even more unknown application potentially than the algal system.

Dr. WARD. I think it would be worthwhile to

point out the design in the creation of that monster started some 3½ years ago.

Dr. MILLER. Yes. Only after extensive experimentation with different types of growth configurations, this is a modular thin panel design where we have a series of algae panels sandwiched in between banks of fluorescent lights; it is a two-man capacity. The total system volume is somewhere in the neighborhood of 100 meters. It has a total exposed surface area of around 28 square meters and a total power requirement of around 30 kilowatts. We do not know whether or not this is inadequate or overly adequate right now.

Dr. REPASKE. We have known for the last 2 years that, in order to sustain one man, at least, with his oxygen requirements, we would probably need a 20-liter culture. It is true that we need smaller cultures originally to work out some of the details, but you always have a scaling-up difficulty. If we work out all of the conditions now for a 2-, 4-, 5- or even 10-liter culture and finally scale up to twenty, we will face additional difficulties. I wonder if it would not be just as easy to get going on all 20-liter cultures and work out the conditions, as well as the difficulties involved in a 20-liter culture and get these out of the way simultaneously, or do we run into other problems?

Since I am not involved in this, it is very easy for me to suggest that we get 20-liter cultures going, but maybe one has to go through these steps slowly.

Dr. FOSTER. We have the 20-liter culture machine built and sitting there waiting either to spend the extra money to buy all of the auxiliary components or tearing down our 2-liter and transferring them. I have hesitated because of control and transfer problems with the 2-liter to shut it down and put them into the 20-liter. From the sense of this meeting, we better shut down and transfer the 20-liter right now.

Dr. SAUNDERS. What sort of volume and weight would that 20-liter carry? The whole system?

Dr. FOSTER. Ours or the flight system?

Dr. SAUNDERS. Yours. The flight system I do not know would be too much different, would it? Weight and volume on it? For 20 liters per day? It is a breadboard, or is it?

Dr. FOSTER. It is a 16-inch cylinder with 24 inches of height. It is a culture vessel and the frame is not as big as the one you have on the 2-liter job.

Dr. DECICCO. It would be weight saving, when you get down to making small units.

Dr. FOSTER. We use the same instrumentation, the same controls and probably the same size gas transfer lines, because we designed into the 2-liter a capability for a 10-fold increase in gas transfer.

Dr. SAUNDERS. Thank you very much. We certainly appreciate the contributions you made and we appreciate you taking the time to come and spend these 2 days with us. Probably the way things are looking now, we will have another one of these meetings—I do not know when—but certainly you will be informed as soon as possible. We will hope that you will again be kind enough to come and join us and give us the benefit of your time and your thoughts.

Thank you all for coming.

Dr. JENKINS. I think the progress has been excellent since last year. I am most pleased to hear what has developed since then.

Dr. SAUNDERS. Again, my thanks to the participants, and I certainly owe a debt of gratitude to the American Institute of Biological Sciences as well as to Mrs. Kaye Walters, my secretary, and Lt. Michael Voorhies, my assistant, for helping us with organizing a successful meeting.

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